HOST MICROFLORA RELATIONSHIP OF VECTORS OF CANINE HEARTWORM DISEASE

By

Dale R. Hamilton

A Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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dedicated to my mother and dad,
whose patience had no limits,

from their son who is less fortunate.

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August, 1975

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Major department: Entomology and Nematology

The phenomenon of early vector mortality of *Dirofilaria immitis* Leidy infected mosquitoes was investigated by means of a bioassay technique. *Anopheles quadrimaculatus* Say were fed differentially filaremic blood meals, living and dead microfilariae, and special adjuncts such as homogenized mosquito body fractions. At least 25% mortality was produced in mosquitoes in the first 3 days following ingestion of living microfilariae. Neither dead, intact, nor homogenized larvae, whole mosquito bodies, nor body fractions reproduced this mortality, suggesting that early vector death is a function of the kinetics of living microfilariae.

An integrated system of diet, technique, and hardware was developed for the production of gnotobiotic blood-feeding arthropods. This system

produced the first successful rearing of *An. quadrimaculatus* under bacteria-free conditions.

A quantitative and qualitative bacteriologic evaluation was accomplished on the midguts of several hundred adult female specimens of colonized *An. quadrimaculatus*, wild *An. quadrimaculatus*, *Anopheles crucians* Weidemann *Anopheles crucians* complex, and *Aedes infirmatus* Dyar and Knab.

The total midgut bacterial count, and the species composition of midgut flora was distinctive for each group of mosquitoes. The most frequently isolated bacterial species for each group of mosquitoes were: *A. infirmatis*, non-pathogenic *Corynebacterium* sp., wild *An. quadrimaculatus*, *Enterobacter cloacae* (Jordan), and *Acinetobacter calcoaceticus* (Beijerinck), colonized *An. quadrimaculatus*, *Bacillus cereus* Frankland and Frankland, *An. crucians*, *Alcaligenes faecalis* Castellani and Chalmers, and *An. crucians* complex, *Salmonella* sp. Data analysis revealed several significant (P .001) positive and negative correlations among the various bacterial species comprising the normal flora of these 5 mosquito groups.

A proportion of bacteriologically sterile specimens were encountered in 4 of the 5 mosquito groups. Sixty-one percent of wild *An. quadrimaculatus* were sterile while 40% of the colonized *An. quadrimaculatus* were sterile. *An. crucians* and *An. crucians* complex were 36% and 43% sterile. All specimens of *A. infirmatus* examined were bacteriologically sterile.

Pure cultures of bacteria isolated from these mosquitoes were used as monocontaminants of gnotobiotic *An. quadrimaculatus*. These mosquitoes as well as conventional and gnotobiotic mosquitoes were subsequently in-

fected with *D. immitis* infective larvae. Forty-eight hours post-infection, all mosquitoes were killed. The degree of filarial development was recorded by light and phase photomicroscopy, and typed according to their gross dimensions.

The early development of *D. immitis* larvae proceed independently of bacteria or bacterial mediation in the midgut. Sausage stage larvae appeared within the first 48 hours in gnotobiotic mosquitoes, but required at least 72 hours in conventional mosquitoes. Nematode development appeared to be marginally improved in the complete absence of bacteria and differentially retarded in the presence of pure cultures of certain bacterial species. Pure cultures of *Lactobacillus* sp. and *B. cereus* were found associated with more advanced larvae, while *Salmonella* sp., *A. calcoaceticus* and *Corynebacterium* sp. monocontaminants were found in the presence of less developed larva. If the presence of midgut bacterial flora is inversely related to the development of *D. immitis* in the mosquito, it is suggested that the "naturally" sterile specimens in the field are the most effective vector of *D. immitis*.

INTRODUCTION

Canine heartworm disease research in the last decade has evolved into two distinct efforts. The first approach has been towards development of adequate chemotherapy to replace the potentially hazardous arsenicals presently in use. Heartworm prophylaxis, although effective, involves considerable risk as well as bothersome clinical management. Development of a vaccine or immunizing agent against canine heartworm disease, although promising (Otto, 1970), is not imminent and must await fuller understanding of the mechanism of immunity to filarial infection.

The second approach, and historically the most successful, is directed against the arthropod vector. *Dirofilaria immitis* Leidy, the etiological agent of canine heartworm disease, depends upon passage through a mosquito to the infective stage. The traditional methods of controlling such arthropod borne diseases such as filariasis, yellow fever, malaria, and trypanosomiasis have been to release sufficient pesticide into the endangered area. This has been justifiable, because it is most certainly DDT and its decendants that have done the most to reduce, and in places eradicate, malaria, typhus, filariasis, trypanosomiasis, and many other of the world's worst killer diseases (Provost, 1972).

In recent years, it has become dramatically apparent that the traditional method of vector control using insecticides is no longer effective. The University of California's Mosquito Control Laboratory recently reported that the common pasture mosquito *Aedes nigromaculis* (Ludlow) is now resistant to every available insecticide (Kendrick, 1970).

Boardman (1973) quoted a report of the Agricultural Experiment Station at the University of California which reported the fact that the two most important species of mosquitoes in California had developed near total insecticide resistance. Elsewhere, the use of organic insecticides against mosquito larvae has led to so much resistance, in *Culex fatigans* Wiedemann especially (Brown, 1967), that control of filariasis and several arboviruses is now one of the greatest problems of vector control. According to a recent review (Busvine and Pal, 1969), resistance to DDT, BHC, and dieldrin is severely affecting control, in one place or another, of malaria, yellow fever, filariasis, typhus, and plague, while certain other vector-borne diseases have so far not been affected by pesticide resistance, e.g., onchoceriasis, Chagas disease, typanosmiasis, and leishmaniasis.

To compound the problem, killing mosquitoes with insecticides is becoming prohibitively expensive. The logic of pest control demands more specific chemicals. However, the more specific a compound is, the more limited its use, and the more costly it is to produce. A decade ago, it cost less than half a million dollars to market a new insecticide. The same development job in 1970 cost from 3 to 5 million dollars (Kendrick, 1970), and today even more. At a time when mosquito abatement districts are demanding new insecticides to replace those of declining value, manufacturers have less incentive to produce them and particularly the narrow spectrum materials designed for limited uses.

The persistance of pesticides in the environment, now thoroughly documented (Woodwell, 1970; Miller and Berg, 1969), has led to increasingly more stringent regulations to govern their uses and application. The Federal Environmental Pesticide Control Act of 1972 for example, established the Environmental Protection Agency which promptly banned the use of DDT.

The net result of increasing insecticidal resistance, obsolete.

pesticides, and legislative restraints is that mosquitoes are increasing in number, and with them, canine heartworm disease. Results of a nationwide geographical survey of canine filariasis taken in 1955, have made it abundantly clear that canine heartworm disease is expanding its range (Young, 1955). The response of 2,337 veterinary practitioners throughout the United States indicated that the distribution of heartworm was primarily in the states bordering the Gulf of Mexico and the Atlantic Ocean, but extending northward to Illinois and central Iowa (Young, 1955). Marquardt and Fabian (1966) collected reports of *D. immitis* in Florida, Georgia, Alabama, Louisiana, Mississippi, New Jersey, Tennessee, Maryland, Arkansas, Pennsylvania, Rhode Island, and Hawaii. Subsequently, enzootic dirofilariasis has been reported in Indiana, Michigan, Ohio, Canada (Otto and Buaman, 1959), Minnesota (Schlotthauer and Griffiths, 1964) and Connecticut (Hirth et al., 1966). In a 1973 statewide survey of Florida, Bradley* reported that 27 north Florida veterinarians diagnosed 4,648 cases of canine heartworm in 1973, an increase of 22% over the previous year. Seventy-seven central Florida veterinarians and 84 south Florida practitioners reported 23,212 and 8,658 cases, respectively, 1973, an increase of more than 50% over the 1972 case level. Eighteen west Florida veterinarians diagnosed 3,927 cases in 1973 which constituted a 33% increase for the year.

Not all mosquito species, however, are capable of transmitting *D. immitis*. Bemrick and Sandholm (1966) list 59 mosquito species known to be successful vectors in the United States. MacDonald (1971) has compiled a list of 107 species in 6 genera of Culicidae which support the development of filarial larvae. Ludlam and Jachowski (1970) report 64 species of mosquitoes in 5 genera in which complete larval development of *D. immitis*

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* R.E. Bradley, Personal Communication, 1973

can take place. Yen (1938) described differences in relative susceptibility to infection in 12 mosquito species. Travis (1947) demonstrated the relative efficiency of 6 species of mosquitoes from Guam, and Intermill and Frederick (1970) reported a similar comparison of Ryuku Island species. Kartman (1952) also reported a quantitative evaluation of the development of *D. immitis* in 7 common mosquito species. Furthermore, evidence has accumulated that indicates that the same species of mosquito may exhibit varying susceptibility to *D. immitis* in different laboratories. Kartman's (1950) monumental survey of the world literature from 1901 to 1949 indicated that the experiments of over 30 workers show that *Aedes aegypti* (Linnaeus) varied in susceptibility to *Wuchereria bancrofti* (Cobbold) from completely inhibiting the parasites' development of allowing maturation of infective larvae.

Even within successful species and refractory species, there are individuals who show varying susceptibility to infection with *D. immitis*. A recent epizootiologic study carried out by Bradley (1971), surveyed Hillsborough County, Florida, an area heavily populated by known vectors of *D. immitis*. Only small numbers of parasites were found in resident dogs, which Bradley (1971), concluded may indicate some factor being exerted on the vector population to limit the exposure of dogs to infective larvae. McGreevy et al. (1970) in a survey of 5 counties in northern California failed to find a single case of *D. immitis* in 515 pound dogs. The same authors surveyed the clinical records of dogs examined for heartworm at the University of California's School of Veterinary Medicine from 1957-1968. Only 13 cases of *D. immitis* infection could be confirmed. Of the known species of mosquito vectors, 9 species were reported to occur in California, and 6 were reported in counties where *D. immitis* was known to occur (McGreevy et al., 1970). A survey of 50 wild foxes trapped within the

geographical limits of a hyperenzootic area in Georgia proved negative for *D. immitis* microfilariae (Walton et al., 1963).

Regional differences in relative susceptibility to infection had also been noted. Roubaud (1937) reported that Assam and Tanganyika strains of *A. aegypti* permitted normal development of *D. immitis* but Cuban strains infected from the same host were entirely refractory. Newton, Wright and Pratt (1945) reported that Puerto Rican strains of *Psorophora confinis* had a 12% infectivity potential for *W. bancrofti*, whereas United States strains had an 80% rate.

The basis of vector susceptibility fo filarial larva has been hypothesized since Nóe (1908) first reported the destruction of microfilariae of *Dipetalonema grassi* Nóe, in the gut of its tick host during the latter stages of feeding. Rozeboom (1973) had observed that filariae exhibit a strict vertebrate host specificity, but the insect vectors for a given parasite may include many species among two or more genera. These parasite-vector associations, he noted, have arisen as the parasites evolved and expanded their geographic distribution and have depended less upon the taxonomic position of the insect species than upon the fortuitous existence of genetically controlled factors for susceptibility. Weinstein (1973) has made the following generalizations regarding the specificity of development of filariae in arthropod hosts.

1. There is generally a higher degree of specificity in the vertebrate phase of the cycle than in the invertebrate.
2. Most recognized vectors are mosquitoes.
3. Filarial development in the arthropod takes place in only one of 3 types of cells (Malpighian tubule, thoracic flight muscle, and fat body), and the specificity of cell type is virtually absolute for a particular species of filariid.
4. Development is intracellular.

A genetic explanation for susceptibility of invertebrate hosts to certain parasites has received substantial support. Huff (1941) determined that the susceptibility of *Culex pipiens* Linnaeus to *Plasmodium cathemerium* Hartman is a recessive character due to a single pair of genes and inheritable along the lines of the classical Mendelian 3:1 ratio. Trager (1942) produced a strain of *A. aegypti* more susceptible to infection with *Plasmodium lophurae* Coggeshall than the original colony strain. A report by the Rockefeller Foundation (1948) indicated that it was possible to raise the susceptibility of *Anopheles quadrimaculatus* Say to *Plasmodium gallinaceum* Brumpt from 20% to 100%. By selective breeding Micks (1949) elevated the susceptibility of *C. pipiens* to *Plasmodium elongatum* Huff and Bloom from 13% to 49% within 6 generations.

Evidence to the contrary had been advanced by Boyd and Russell (1943) who could obtain no clearly defined results in attempting to select a strain of *An. quadrimaculatus* more susceptible to *Plasmodium vivax* (Grassi and Feletti). Jeffrey (1949) similarly failed to produce a strain of *Anopheles albopictus* (Skuse) more susceptible to infection with *P. lophurae* and Horvanitz (1947) could obtain no genetic effects in 6 generations of *A. aegypti* selected for susceptibility to *P. gallinaceum*.

A genetic basis of mosquito susceptibility to filarial nematodes was first suggested by Roubaud et al. (1936) and Roubaud (1937) who noted differences in the susceptibility rate between colonies of *A. aegypti* originating from different geographical areas and exposed to the same strain of *D. immitis*. Ramachandran et al. (1960) reported similar variations in susceptibility of *A. aegypti* for *Brugia malayi* (Brug). The response of a colony of mosquitoes to artificial selection pressure for changed susceptibility was noted by Kartman (1953a) with *D. immitis* and by Thomas and

Ramachandran (1970) for *Wuchereria* sp. in *Culex pipiens fatigans* Wiedman.

MacDonald (1962a, 1962b, 1963a, 1963b) determined that susceptibility to infection of *A. aegypti* with sub-periodic *B. malayi* was controlled by a sex-linked, recessive gene called F^M which exhibited incomplete dominance. Subsequently, MacDonald and Ramachandran (1965) reported that this gene controlled development of 5 strains of *Wuchereria* sp. and *Brugia* sp. in the thoracic muscles of *A. aegypti*, but apparently had no effect on the development of *D. immitis* or *Dirofilaria repens* Raillet and Henry in the Malpighian tubules. McGreevy (1972) finally demonstrated that susceptibility of *A. aegypti* to *D. immitis* was also controlled by a sex-linked, recessive gene and that penetrance of this gene was incomplete. Moreover, the gene that controls the development of *D. immitis* in the Malpighian tubules is not identical to the F^M gene which controls the development of *Wuchereria* sp. and *Brugia* sp. in the thoracic muscles. MacDonald (1973) cited unpublished work by Oblamine and MacDonald that indicates the susceptibility of *C. p. fatigans* to *B. pahangi* was also controlled by a sex-linked, recessive gene.

Kartman (1952) considers that genetic factors may operate independently of the phylogenetic position of the host. The efficiency of 2 closely related mosquitoes as vectors for *D. immitis* is not necessarily comparable. Instead, susceptibility seems to be an inherent characteristic of the individual mosquito. Hu (1937) gave *C. p. pallens* double spaced infectious feedings of *W. bancrofti* in an effort to determine whether susceptibility to infection with filaria in an inherent characteristic of the individual. He reported that some of the females were susceptible to the first but not to the second infective meal, and vice versa. Similarly, immunity to reinfection with *D. immitis* would not develop in *Anopheles punctipennis* (Say), *Aedes cinerus* Meigen, and *Aedes triseriatus* Say (Phillips, 1935). Kartman

(1953) noted an unusually high susceptibility for *D. immitis* among certain individuals of Hawaiian strain of *A. aegypti*, a species generally refractory to this filariae. Furthermore, individual respone of a mosquito host is distinctly specific to the species of parasite. Kartman (1952) could demonstrate no correlation between susceptibility of *A. aegypti* to mixed infections of *D. immitis* and *Foleyella* sp. Each species of filaria reached its normal location within the mosquito without interference from the other species. This is consistent with the observations of Huff (1934) on infection of *C. pipiens* with several species of avian plasmodia, and with those of Moorthy (1938) on the development of *Canallanus* sp. in cyclops previously infected with *Dracunculus* sp.

A more critical examination of the individual mosquito, rather than a species as a unit, seems to be warranted. The factors and circumstances which serve to distinguish the individual mosquito are manifold, and may have an important effect on the individual's susceptibility to many parasitic agents. One of these distinguishing factors may be quantitative and qualitative differences in the composition of intestinal microflora.

Mosquito species tend to select very specific nesting sites and subsequent rearing sites for the larvae. Considerable diversity exists between species in their choice of sites which range from vernal ponds (Wills and Fish, 1973), pools and flood waters (James and Harwood, 1969) to tree holes and automobile tires (Womack, 1971), and in certain water holding plants such as bromeliads, heloconids and pitcher plants (Gillette, 1972). The bacterial and fungal populations of these selected microhabitats reflect the diversity of their environment.

Newly hatched mosquito larvae are indiscriminant filter-feeders which voraciously injest anything of a specific particle size. Since this

particle size is usually less than 100 microns, bacteria make up a substantial proportion of the larval diet. This fortuitous acquisition of microflora apparently established the individual's lifetime complement of internal bacteria, which is both species specific and individually distinct. Qualitative and quantitative differences in the composition of this microflora may represent one of the factors mediating vector susceptibility to filarial infection. The passage of filarial larvae into and through the gut of susceptible mosquitoes evidently requires a critical set of conditions since attempts to culture infective filaria larvae have met with little success (Weinstein, 1970, 1972; Rothstein and Brown, 1960; Sawyer and Weinstein, 1953a, 1953b; Taylor, 1960b; Wood and Suitor, 1966; Cupp, 1972). Resident microflora may indirectly interact with the infective larvae, or merely mediate an intestinal climate favorable to survival of the parasite.

The demonstration of an interaction between larvae of *D. immitis* and resident bacteria, may provide a future means of biocontrol for both anthropod and nematode.

LITERATURE REVIEW

Subsequent to feeding on a filaremic dog, 3 stages in the development of *D. immitis* occur within the mosquito (Taylor, 1960). A moult marks the onset of each stage terminating in the third stage infective larva. Microfilariae taken in with the blood remain in the stomach of the mosquito for the first 24 hours. During the next 24 hours they migrate to the Malpighian tubules. Further development occurs at the distal end of the tubules until the fifteenth or sixteenth day. The larvae spent the first 6 or 7 days inside the cells of the Malpighian tubules. Suguri et al. (1969) have described this zone on the Malpighian tubules as rich in mitochondria and coarse endoplasmic reticulum. The brush border has a multitude of vertically arranged protoplasmic processes each containing a mitochondria whose cristae run parallel to the process. From the ninth to the fifteenth day, larvae are found in the lumen of the tubules. By the fifteenth day the larvae are ready to break out of the tubules and migrate through the mosquito, to occupy the cephalic spaces of the head and proboscis (Taylor, 1950). Transmission of infective larvae occurs with the next blood meal. Although many arthropods have been evaluated as vectors of *D. immitis*, only mosquitoes appear to be entirely competent (Ludham and Jachowski, 1970).

Kartman (1952) observed that although microfilariae of *D. immitis* are known to be capable of metamorphosis in many species of mosquitoes, there is a distinct paucity of host species in which it may adequately reach the infective larval stage. The same writer introduced the concept of "host efficiency," a ratio between theoretical numbers of microfilariae ingested

by a group of mosquitoes and the total number of developing larvae found in them during a specified period, and "infective potential," a similar ratio involving only infective larvae. Kartman (1952) reported that the host efficiency of *An. quadrimaculatus* was approximately 20 times better than either *A. aegypti* or *Culex quinquefasciatus* Say; and that the infective potential of *An. quadrimaculatus* was 50 times greater than *A. aegypti* and 20 times more than *C. quinquefasciatus*. Infection of *C. pipiens* and *C. quinquefasciatus* showed that both the host efficiency and the infective potential of *C. quinquefasciatus* were 2-1/2 times greater than those for *C. pipiens*. Ewart (1965) correlated variations in vector potential of *Culex*, *Anopheline*, and *Aedes* mosquitoes to failure of *Brugia pahangi* Buckley microfilaria to exsheath. Weatherby et al. (1971) reported a similar case of host efficiency in susceptibility to *P. gallinaceum* by feeding mosquitoes whole body extracts of both susceptible and refractory mosquitoes. *Culex pipiens* extract (refractory) fed to susceptible *A. aegypti* reduced the number of oocysts developing by 50% while concentrations of 75% extract reduced the numbers to only 34% of the number in the controls. Clearly, some factor present within refractory mosquitoes, and transferable, was capable of reducing the host efficiency of susceptible mosquitoes.

A problem fundamental to the production of experimental *D. immitis* infections has been survival of the mosquito following uptake of microfilariae from infected canine blood. Various authors including Yen (1938), Kershaw et al. (1953), Bradley (1953a, b), Husain and Kershaw (1966), Webber and Hawking (1953), Pistey (1959), Symes (1960) and Weiner and Bradley (1970) have reported vector mortality approaching 100% in the first few days following the blood meal. A common antecedent to death appears to be distension of the abdomen persisting 2 to 3 days, which Weiner and Bradley (1970) interpret

as a result of the improper digestion of blood. Additionally, it appears that vector mortality increases proportionately with the number of microfilariae ingested (Rosen, 1954; Kershaw et al., 1955; Wharton, 1957; Kartman, 1952). Kartman (1952) suggested that variations in vector survival were sometimes dependent upon genetic strains of the same species, and later (1953a) observed a direct relationship between numbers of microfilariae fed to mosquitoes and vector survival. Diet (LeCorroler, 1957), environmental temperature and humidity (Galliard, 1957), but not atmospheric pressure (Williams, 1959) have been shown to affect survival of filarial-infected mosquitoes. Laurence (1963) had estimated the natural mortality of 2 infected mosquito populations in the field. *C. fatigans* and *Anopheles peditaeniatus* (Leichester) in south India had a daily mortality of from 14% to 24% during a season favorable for survival.

Kershaw et al. (1953) investigated the effect of microfilaria of *D. immitis* on the longevity of *A. aegypti* under controlled laboratory conditions. Infected mosquitoes had a constant but very low survival rate during the first 5 days corresponding to the migration of microfilaria from the midgut to the Malpighian tubules of the mosquito: a constant and high survival rate from the fifth to the twentieth day, corresponding to the development in the tubules; and a very low but constant survival rate from the 21st to the 25th day, corresponding to the presence of infective larvae in the hemocoel and in the head and mouthparts of the mosquito.

Septicemia induced by migrating microfilaria may be another contribution to vector mortality in the first few days following infection. Intestinal microflora, fortuitously acquired in the larval stage, are unable to multiply in the conditions prevailing in the gut of healthy insects (Bucher, 1960). Substantial mortality from bacterial infections in laboratory colonies had

been reported when environmental conditions favor the multiplication of one or more of the bacterial species already present in the gut (Prinsloo, 1967). Chu and Toumanoff (1966) reported that most of the bacteria isolated from laboratory colonies are Gram-negative which Kalucy and Daniel (1972) concluded are "potential pathogens" which require predisposing factors such as damage to the cuticle or gut wall in order to penetrate the hemocoel. The bacteremia resulting from multiplication in the hemocoel caused death of the host.

Nematode-introduced septic conditions are not unknown in the literature. Most of the infective juveniles of the DD-136 strain of *Neoaplectana carpocapsae* Weiser contain cells of a specific bacterium, identified and described as *Achromobacter nematophilis*, in the ventricular portion of their intestinal lumen (Poinar, 1972). Soon after the nematode reaches the hemocoel of a parasitized insect, such as *Galleria* larvae, the bacterial cells are passed from the intestine out through the anus into the hemolymph. The bacteria then rapidly multiply in the hosts' body causing a fatal septicemia. In most instances it is the bacteria that actually kills the host-usually within 48 hours; however, most nematodes without their associated bacteria are also able to kill the host within a short period. Without the *Achromobacter* bacteria Poinar (1972) reports, *N. carpocapsae* is unable to gain access into the insect hemocoel, thus the relationship between *A. nematophilis* and *N. carpocapsae* must be considered mutalistic (Poinar and Thomas, 1967). Such bacteria are undoubtedly associated with all of the 11 known species of *Neoplectana* (Welch, 1965).

Large numbers of microfilariae ingested with blood meal have been shown to interfere with the formation of the peritrophic membrane with fatal results (Lewis, 1953). Wanson (1950) noted that the blood contained in the stomach of *Simulium damnosum* Theobald killed by heavy microfilariae

infection, appeared to be undigested. Similar observations were recorded by Roubaud et al. (1933) and Mackerrar (1953) on other filariae-host combinations. Lavoipierre (1958) regarded the most likely cause of death in heavily infected arthropods as "indigestion" due to extensive damage to the peritrophic membrane rendering the digestion of blood almost impossible. The same author found it impossible to demonstrate the peritrophic membrane in dying heavily filaremic *A. aegypti* with undigested blood meals, whereas the membrane was present in the controls in which the blood meal was in an advanced state of digestion. Bain and Philippon (1970) found the peritrophic membrane intact in *Anopheles stephensi* Liston and *S. damnosum* given a blood meal containing reptile microfilariae. Furthermore, they concluded that the peritrophic membrane did not prevent the crossing microfilariae from the blood meal to the midgut. Lewis (1953) reported that peritrophic membrane formation in *S. damnosum* following the blood meal was critically important to the survival of *Onchocerca volvulus* Leuchart. Only the filariae which remain in the anterior portion of the midgut are able to pass backward (after formation of the membrane) into the space between the membrane and the gut wall in order to pierce the stretched walls of the distended stomach.

Wigglesworth (1965) described two different methods of peritrophic membrane formation around the food bolus. The membrane may be delaminated from most or all of the midgut epithelial cells, or it may be secreted from specialized cells in the anterior midgut. The midgut epithelium of mouse-fed *A. aegypti* produces a granular secretion for up to 15 hours after feeding and the peritrophic membrane is apparently formed from this (Bertram and Bird, 1961). The membrane formed by the first method generally acquires the shape of the food, bolus, while that formed by the second method is usually cylindrical. In larval mosquitoes, the membrane is formed by the

second method (Wigglesworth, 1930; Richards and Richards, 1971). The membrane in adult mosquitoes is formed by the first method (Freyvogel and Staubli, 1965; Freyvogel and Jacquet, 1965; Stoher, 1957). Romoser and Rothman (1973) published the first report of the presence of peritrophic membrane around the meconium of a pupal mosquito. Romoser (1974) subsequently demonstrated a second membrane, closely applied to the first which is formed somewhat later in the pupal period. Howard (1962) reported that the peritrophic membrane appears about 12 hours after *A. aegypti* feeds, and increases in size up until 36 hours, and becomes brittle by 48 hours. As the amount of blood in the midgut decreases, the peritrophic membrane is fragmented by the contraction of the midgut muscles.

Zhuzhilov (1962) reported that the peritrophic membrane was apparent in *A. aegypti* within 20 minutes of taking a blood meal. In contrast, Freyvogel and Staubli (1965) could not discern the membrane in this species before 8 hours after feeding. Richardson and Romoser (1972) reported that the membrane was visible in *A. triseriatus* 20 minutes after taking the blood meal.

Stohler (1957) noted that the membrane adheres to the blood parcel, not to the midgut cells. Although the membrane in *Aedes* sp. passes through stages described by Freyvogel and Staubli (1965) as viscous, elastic, solid, and finally fragile, the membrane in *Anopheles* sp. never develops beyond a fragile membrane. The same authors also reported that 3 species (*A. aegypti*, *Anopholes gambiae* Gilas, *An stephensi*) which normally form a peritrophic membrane do not do so completely if they ingest only a small quantity of blood. Richards and Richards (1971) found no trace of a membrane in adult male or unfed adult female *A. aegypti* 1 to 3 days post emergence. Older adult females given a blood meal produce a peritrophic membrane from material

secreted by a ring of midgut cells in the anterior half of the proventricular pouch.

The rate of destruction microfilariae during the first 48 hours after ingestion determines vector mortality to a considerable extent. Hughes (1950) observed that microfilaria of *Litomosoides* ingested by *Ornithonyssus bacoti* (Hirst) mites were phagocytosized by certain cells in the midgut. Kartman (1953) regarded the initial destruction of microfilariae in the midgut as a process of digestion, although, as he pointed out, death may not be caused by digestive enzymes but rather by other factors in the midgut or salivary secretions. Digestive destruction of inappropriate parasites is an early hypothesis to explain vector-parasite specificity (Nuttall, 1908) which remains an attractive explanation. Digestion of the blood meal by mosquitoes begins at the outer edge of the meal and proceeds inward (Davis and Phillip, 1931). This has been observed in *C. pipiens* (Huff, 1934), *A. aegypti* (Stohler, 1957), *An. stephensi*, *An. gambiae* and *Anopheles maculipennis* Meigen (Freyvogel and Staubli, 1965). Before engorgement they become squamous with convex internal borders (Howard, 1962). Bertram and Bird (1961) observed in the midgut cells of *Aedes* after a blood meal, that whorles of granular endoplasmic reticulum condensed around the nucleus unfold to form a complex system "ramifying throughout the cytoplasm" which revert to whorles when blood digestion is complete. These changes, confirmed by Staubli et al. (1966), apparently facilitate the liberation of proteolytic enzymes for digestion of the blood meal, and Hector and Freyvogel (1971) noted that whorl formation was found only in female mosquitoes.

The anterior midgut cells of *An. gambiae* secrete fairly large amounts of mucous-like material within 7 minutes of a blood meal. Although this mucous forms a plug at both ends of the stomach and often completely surrounds

the meal, Freyvogel and Staubli (1965) have concluded that it exerts no important effects on digestion.

The presence of a microfilaricide in the digestive secretion of the mosquito host has yet to be verified. Duncan (1926) determined the bactericidal activity of the gut contents and feces of several bloodsucking arthropods including *Anopheles bifurcatus* (Linnaeus), *A. cinereus*, *Stomoxys calcitrans* (Linnaeus) and blood fed *Musca domestica* (Linneaus). Activity was found against 8 of 18 species of bacteria used. The bacterial material from *S. calcitrans* was heat stable (100°C for 30 minutes) and was not destroyed by trypsin. St. John et al., (1930) found no evidence of bactericidal material in the digestive tract.

Kartman (1952) also considered that the spped of microfilarial passage through the midgut to be of critical importance to larval survival, and was probably related to the presence or absence of salivary anticoagulants. Salivary anticoagulants have been demonstrated in *An. maculipennis* (Yorke and Macfie, 1924), *An. quadrimaculatus* and *An. punctipennis* (Metcalf, 1945), *Anopheles subpictus* Grassi (Cornwell and Patton, 1945). They are absent in *A. aegypti* (Metcalf, 1945), *Aedes dentritus* Haliday (Shute, 1935), *An. stephensi* (Shute, 1948), *C. quinquefasciatus* and *C. pipiens* (Shute, 1936). Salivary anticoagulins appear to prevent the premature clotting of the blood meal (Lloyd, 1928).

Salivary agglutinins are absent in *A. aegypti*, *A. dentritis* (Shute, 1935), *An stephensi* (Shute, 1935), and *C. pipiens* (Shute, 1936). Salivary agglutinins have been demonstrated in *An. maculipennis* (Shute, 1948), *An. quadrimaculatus* (Metcalf, 1945), and *An. subpictus* (Cornwell and Patton, 1914). It should be noted that salivary agglutinins from *An. quadrimaculatus* are effective against red cells of man, mule, cow, pig, dog, rabbit,

and mouse, but not chicken or turtle (Metcalf, 1945).

The addition of an anticoagulant to the infective blood meal produces a rate of microfilarial migration in *A. aegypti*, a poor vector, which is quite comparable to that in *An. quadrimaculatus*, an efficient host (Kartman, 1953). Zaini et al. (1961) reported 91% of *B. malayi* microfilariae ingested by *Aedes (Finlaya) togoi* (Theobald), which lacks an anticoagulin, were trapped in the stomach blood clot after 24 hours.

The speed of blood digestion by the mosquito may affect microfilaria survival and is often quite variable between species. Variations in digestive rates are related to such factors as photoperiod (O'Gower, 1956), temperature (Sella, 1920), season (Guelmino, 1951), and the amount of blood ingested (DeBoissezon, 1930). De Buck et al. (1933) suggested that refractoriness of *An. maculipennis* to *Plasmodium* was correlated with slow digestion of the blood meal in certain varieties during overwintering.

Thayer et al. (1971) have advanced the interesting hypothesis that host specificity of the insect and its ability to transmit certain parasites may be related more to components of the blood meal itself rather than differences in digestive power. In a comparison of the free amino acid levels following a human blood meal to those after an avian blood meal revealed significant differences in the concentrations of aspartic acid, isoleucine, and carinosine.

One of the most consistently demonstrated phenomena regarding the resistance of mosquitoes to invading microfilaria is that of encapsulation, a process by which the parasite becomes impregnated with a brown deposit which eventually forms a capsule. Encapsulation or chitinization, is currently viewed as a defense mechanism (Salt, 1957), which may occur around living or dead microfilaria (Kartman, 1950). Yen (1938) reported

encapsulation of all larval stages of *D. immitis* in the stomach, Malpighian tubules, and the body cavity of mosquitoes. Schacher (1962) reported the recovery of encapsulated larval *B. pahangi* from *An. quadrimaculatus* and *P. confinis*.

Salt (1955) reported two defense reactions of *Microlepidoptera* against experimentally injected parasitoid eggs, which consist of encapsulation and the deposition of melanin. The deposition of melanin, a well-known reaction of the tyrosinase complex of enzymes on tyrosine, or an intermediate chromogen in the presence of oxygen, acts as defense reaction only fortuitously; when the deposit is so cited as to prevent a vital activity, such as the hatching or the feeding of the parasite (Salt, 1957). A similar reaction was noted in parasitoid-infected *Carausius* in which Salt (1956) described the melanin reaction to be as strong in confined spaces as in the main blood stream and progressing to encase the parasite in a brittle black sheath. Salt (1960) described a rapid reaction of the haemocytes of the tomato moth caterpillar against infected parasitoid eggs or larvae, which led to encapsulation within 4 hours and the subsequent death by asphyxiation of encapsulated parasites. The haemocyte reaction was similarly invoked by other species of parasites, by dead parasites, by glass rods, pieces of nylon thread, by injured pieces of the caterpillar itself, and by organs transplanted from other species of caterpillar. Salt (1960) concluded that surface properties of the parasite or implant determine whether or not it will be encapsulated. Goodwin (1958) previously noted that the action of diethylcarbamazine on microfilariae and diaminodiphenoalkane derivatives on schistosomes was "to change the surface of the parasite in wuch a way that it becomes recognizable to the host as an intruder."

Bronskill (1962) noted an identical reaction in the larvae of *A. aegypti*, *Aedes stimulans* (Walker), and *Aedes turichuris* (Dyar), when rhabditoid DD136 juveniles (an undescribed species of the Neoaplectanidae with its associated bacterium) penetrated the blood sinus and entered the body cavity. By 5 hours, a thick capsule developed about many of the ensheathed immature forms as a result of a rapid defense reaction of the mosquito which had both a melanin and a cellular manifestation. He further noted that this type of host resistance resembles that of adult mosquitoes to filarial nematodes. Poinar and Leutenegger (1971) have recently shown that this reaction in mosquitoes is a humoral type of melanization and unlike typical melanization reactions in insects, does not depend on the direct presence of hemocytes as a precursor stage of melanization. They describe a deposit which slowly became pigmented, orginated from components in the noncellular portion of the hemolymph that coagulated out on the surface of 4 neoplectanid nematodes in *C. pipiens*.

Whether the "gut barrier to infection" is due to the enzymes of the digestive processes or to mechanical factors of movement through the alimentary tract remains unknown. Chamberlain and Sudie (1961) have advanced several hypotheses including virus inactivation by digestive fluids and the impermeability of the peritrophic membrane, but emphasize that no mechanism has been completely proven. Lavoipierre (1958) reviewed the relationships between filarial nematodes and their arthropod vectors including a brief discussion of the possible role of digestive physiology of the vector in limiting the intensity of infection.

The endocrine physiology of the arthropod vector has also been implicated in the mediation of filarial competence. The insect endocrine system is remarkable in its complexity and its ability to integrate development and

physiological events with the environment. The parasitic nematode must respond appropriately to this changing milieu of hormones because they reflect the external environment in which the host finds itself. Davey and Hominick (1973) observed that where the nematode-arthropod association is obligatory, the long evolutionary history of host and parasite may have resulted in a dependence of the nematodes on the endocrines of the host. They suggest that such a dependence might take the form of host endocrines providing cues to the nematode to sense the hormone molecule itself; an indirect cue would have the nematode respond to a hormone induced change in the physiology of the host.

Numerous reviewsof insect endocrinology have appeared recently in the literature (Highnam and Hill, 1969; Wigglesworth, 1964, 1970). At least 6 endocrine tissues have been described in insects. Neurosecretory cells of the pars intercerebralis of the brain have axons which terminate in the corpus cardiacum, a storage and release organ closely associated with the heart. These cells secrete "activation hormone" which activate the thoracic (prothoracic) gland (Wigglesworth, 1970), and also a factor that is involved in the control of egg formation (Davey, 1965), protein synthesis, and the mobilization of lipids (Wigglesworth, 1970). The hormonal cycle in bloodsuckers is initiated by distention of the midgut wall following engorgement (Bertram and Bird, 1961), which stimulates the neurosecretory cells in the brain to release a hormone into the hemocoel (Lea, 1967). This in turn stimulates the corpus cardiacum to secrete the gonadotropic hormone; oocysts begin micropinocytosis (Roth and Porter, 1964; Anderson and Spielman, 1971), vitellogenin is synthesized (Hagedorn and Judson, 1972), and eggs subsequently mature. The corpus cardiacum also contains its own intrinsic cells which produce and release a variety of other hormonal factors chief among them a

peptide active in the control of heart and other muscles (Davey, 1964).

The paired corpora allata produce juvenile hormone (Wigglesworth, 1954, 1964, 1970) which is active in development, and is essential to the full expression of egg production. Juvenile hormone determines the direction of the development initiated by ecdysone; whether toward another larval form, a pupal form, or the adult form (Wigglesworth, 1954, 1964, 1970). It also governs the titre in the hemolymph of the yolk protein by regulating its synthesis in the fat body (Engelmann, 1969). Juvenile hormone has been isolated from a wide variety of plant and animal sources (Schneiderman and Gilbert, 1958; Williams, Moorhead and Pulis, 1959). Schneiderman, Gilbert and Weinstein (1960) reported that cell-free broth of *Escherichia coli* and whole broth from *Proteus* species had a very high juvenile hormone activity in the polyphymus test.

The principle remaining endocrine organ, is that thoracic gland which through the production of ecdysone or moulting hormone, initiates those developmental events culminating in the production of ecdysone or moulting hormone. It also initiates those events culminating in the production of a new cuticle and the shedding of the old. Although insect gonadal tissue has been considered as nonendocrine, in some insects at least, the testis (Naisse, 1966), and the ovary (Pratt and Davey, 1972) have been shown to produce hormones. Even the hindgut has been implicated as a source of hormones in at least 1 species (Beck and Alexander, 1964).

The role of hormones in mosquitoes is of special interest since Bhattacharya and Chowdury (1964) reported that certain vertebrate steroids appeared to increase the susceptibility of *A. aegypti* to infection with *W. bancrofti*. Spielman and others (1971) reported that 1 steroid hormone ecdysterone, stimulates ovarian development in adult mosquitoes, an effect

not observed after the administration of various vertebrate steroids. However, administration of massive quantities of ecdysterone did not influence the development of *B. pahangi*. Gwardz and Spielman (1974) reported that application of synthetic ecdysone and juvenile hormone to *A. aegypti* did not affect the development of *B. pahangi*.

Certain oxyurid parasites of roaches appear to require hormones from the hosts' corpora allata (Nadakal and Nayer, 1968), and median neurosecretory cells (Gordon, 1968, 1970) for development. Even nonentomophilic nematodes may be influenced by the presence of such exogenous insect hormones as juvenile hormone and ecdysterone (Hitcho and Thorson, 1971; Johnson and Viglierchio, 1970; Meerovitch, 1965; Shanta and Meerovitch, 1970; Webster and Craig, 1969; Davey, 1971).

Davey and Hominick (1973) have concluded that however attractive the notion that development of a parasitic nematode is under the control of the host endocrine system may be for many authors, the cases in which this has been demonstrated are very rare. Yoeli and others (1962) found that microfilariae of *D. immitis* developed to the sausage stage in *A. quadrimaculatus* decapitated immediately after the infective blood meal. Since the vitellogenic hormone is secreted and stored in the head of the mosquito, this finding established that the initial stages of larval differentiation are not controlled by the same hormone system that regulates host oogenesis. Gwardz and Spielman (1974) showed that the allatum secretion does not influence the development of larval *B. pahangi*. Such larvae mature in allactomized juvenile-hormone "free" females of both susceptible and refractory mosquito species. These authors also concluded that larval development of *B. pahangi* was independent of the insect hosts' endocrine milieu.

Dual Etiology of Parasitic Diseases

The relationship of normal intestinal microbiota to the vectoring capacity of insects is an idea that has not yet been adequately investigated. Insects are not particularly unique among animals in their relationships involving bacteria and fungi. Parasites passing through the host's alimentary tract are exposed to both the microorganisms that reside there and to the intestinal climate which may, in part, be influenced by them. Indigenous microorganisms present at the host-parasite interface must be regarded with as much consequence as the host itself.

Gnotobiotic studies have shown that the pathology of many parasitic diseases depend not only upon the etiological agent, but also upon the host's intestinal microflora. Phillips and Gorstein (1966) have reported that the association of ameba *Entamoeba histolytica* Schaudinn with living bacteria such as *Escherichia coli* (Migula), *Aerobacter aerogenes* (Kruse), and *Clostridium perfringens* (Vellon and Zuber) was necessary to produce typical hepatic abscesses in hamsters. Wittner and Rosenbaum (1970) confirmed this study and observed that the participation of bacteria with ameba must be more than just maintenance of a suitable intestinal environment, but might involve a resistance transferring factor described by Anderson (1965) passed from bacteria to ameba. Phillips (1964) considered that the bacterial flora acted by "providing a suitable environment, physical and chemical, for excystation and establishment of lumen infection until such time as the ameba can enter the tissue."

Infectious enterophepatitis in turkeys has been shown to reflect a dual etiology requiring *Histonomas meleagridis* (Smith) and a single species of bacteria (*E. coli*, *C. perfringens*, or *Bacillus subtilis* Cohn) (Bradley et al., 1964; Bradley and Reid, 1966). Franker (1965) produced a moderate rate of

infection with this protozoan and a species of infection with this protozoan and a species of *Eimeria* in combination with *Bacillus cereus* Frankland and Frankland or *Lactobacillus fermenti* Beijerinck. Springer et al. (1970) noted that the bacterial requirements for producing this disease in bacteria-free chickens were different from those for the disease in bacteria-free turkeys. The essential contributing factor of bacteria in the pathogenesis of infectious enterohepatitis was determined to be neither a favorable pH, nor an oxidation-reduction potential value within the intestine of the host, but rather th make the cecal environment more suitable for survival of the transport host, *Heterakis gallinarum* (Schrank).

Wescott (1971) noted in gnotobiotic mice infected with *Nematospiroides dubius* Baylis and *Nippostrongylus brasiliensis* Travassos that more parasite developed, infections were of greater duration, and of a higher reproductive potential in the conventional rather than in germ-free hosts.

Feline infectious enteritis was shown by Johnson and others (1967) and Rohovsky and Griesemer (1967) to be almost asymptomatic in germ-free cats, but severely symptomatic in specific-pathogen-free (SPF) cats. Visco and Burns (1972) reported a parallel situation in gnotobiotic chickens infected with *Eimeria tenella* (Railliet and Lucet). It was previously demonstrated by Radhakrishnan (1971) that *E. tenella* could produce typical cecal pathology in gnotobiotic chicks only when associated with certain bacteria or combinations of bacteria. Johnson (1971) noted severely depressed growth and development of *Ascaridia galli* (Schrank) in germ-free chicks and Bosmak (1971) reported that *Hymenolepis nana* (von Siebold) in mice appears dependent upon host bacteria, and that the variety of bacteria present changes with increasing intensities of tapeworm burden. Conversely, the absence of bacterial associations may be a factor in disease transmission. For

successful transmission of leishmania, the *Phlebotomus* fly must have a sterile alimentary tract because human leishmanias do not tolerate bacterial contamination. Infection of *A. stephensi* mosquitoes with a microsporidian parasite *Nosema algerae* Vavra and Undeen, 1970, reduces their susceptibility to infection with the simian malarial parasite, *Plasmodium cynomolgi* Mayer as measured by malarial oocyst counts 6 days after the infective meal (Ward and Savage, 1972). Mortality from nosematosis was so great that 90-95% of the exposed mosquitoes died by sporozoite levels could be assessed. Fox and Weiser (1959) pointed out that there is apparently no evidence that *Nosema* is physiologically antagonisitc to plasmodium, or that the former attacks the latter. These investigations concluded that the midgut wall of heavily infected mosquitoes was so disintegrated that suitable sites were not available for oocyst development. From these and similar studies, it is apparent that the intimacy of host bacterial flora with parasites appears to be more significant than circumstantial.

Microflora Associated with Mosquitoes

Mitchell's (1907) report that larval *A. aegypti* were predominantly bacteria feeders was based on the observation that larvae develop more rapidly in water contaminated by sewage. The same year Putter (1907) reported that organic colloids and solutes serve as the primary food source for aquatic invertebrates. Hinman (1930, 1932) and Trager (1936) considered that various substances in solution served as the sole food source, but Shipitzinia (1930), and Rozebloom (1935) and Buddington (1941) showed that pond water passed through a bacterial filter could not support growth beyond the fourther instar. Trager (1935), however, reported that larva could

utilize liver extract and whole killed yeast in solution.

Lamborn (1921) claimed that some mosquito larvae feed on specific living organisms such as *Spirogyra* sp., *Euglena* sp. and *Volvox* sp., but Metz (1919) showed that it made little difference whether a variety of animal or vegetable matter fed to anopheline larvae was living or dead. Hinman (1930) reported that bacteria, algae, and protozoa although ingested by mosquito larvae, were not digested and hence, contributed little to the nutrition of larvae.

Laird (1956) concluded that algae and protozoa are the primary sources of food for mosquitoes breeding in permanent ponds and lakes in mosquito breeding areas of the South Pacific. Coggeshall (1926), Hamlyn-Harris (1928), Senior-White (1926), and Howland (1930) have all reported that algae is the primary food source of at least some mosquito species.

Atkin and Bacot (1917) raised larvae of *A. aegypti* in pure cultures of *Saccharomyces cerviciae* Meyen and *Bacillus coli communior* (Durham) and Bacot (1917) noted that mosquito larvae kept in water originally turbid with bacteria, was rapidly cleared. The intestinal contents of such larvae were relatively free of bacteria, which Bacot (1917) considered a result of complete digestion. Glasser (1924) considered that liver and yeast supplied certain accessory growth factors to the larvae of the several species of flies he maintained in culture. Bragina (1926) nourished newly hatched *A. maculipennis* on bacteria (unidentified) and Barber (1927) found that algae alone, bacteria alone, or infusoria alone constitutes a sufficient food source for *Anopheles crucians* and *A. quadrimaculatus*. These infusorians were chosen as representative of the organisms commonly found in the plankton of *Anopheles*-producing water and included: *Colpidium* sp. isolated from cultures of rotting algae, a motile, unicellular, grass-green algae, possibly

Chlamydomonas sp. or a related form; and a large and a small variety of *Spirillum* sp. Barber (1928) reported similar results using these organisms as food for culicine larvae.

Rozeboom (1935) reported that bacteria, to a certain extent, can be utilized as food by *A. aegypti*, *C. pipiens*, *Culex territans* Walker and *Culex salinarus* Coquillet larvae. Environmental bacteria, associated with the different natural breeding places of the mosquitoes, promoted optimum development when bacteria were the only source of food. Rozeboom (1935) found that *E. coli*, *Bacillus subtilis* Cohn, *Bacillus mycoides* Flügge, *A. aerogenes*, and *Pseudomonas aeruginosa* Migula were of equal value, but that *Sarcina lutea* Schroeter was inferior. *Pseudomonas fluorescens* Migula had a toxic effect on young larvae. Bridges (1965) reported that *B. subtilis*, *B. megatherium*, and *Staphylococcus aureus* Rosenbach were unsatisfactory as a sole source of food for *C. quinquefasciatus* larvae. The yeast *S. cerevisiae* provided the best food source. *E. coli* and *A. aerogenes* were about equally indifferent in food value. *P. aeruginosa* and *S. lutea* were utilized as food only to a limited extent. Buddington (1941) reported that *E. coli*, *B. subtilis*, and *Bacillus megatherium* DeBary in Berkefeld-W filtered pond water supplied sufficient nutrients for the complete development of *A. aegypti*. Beattie and Howland (1929) considered that bacteria play some part in the nutrition of *Orthopodomyia* sp. and Rudolfs and Lackey (1920) reported that microorganisms from decomposed vegetable matter were responsible for mosquito breeding. Laird (1956) concluded that bacteria were the primary food of rain pool mosquitoes because bacteria were the most abundant organisms growing in these transient ponds.

Yeasts have been shown to contain most of the nutrients needed by mosquitoes for complete growth (Atkin and Bacot, 1918; Trager, 1935; Frost

et al., 1936; Subbarow and Trager, 1940; Buddington, 1941, Goldberg and de Meillon, 1948; Akov, 1962). Alcohol sterilized, but not heat sterilized yeast will support complete development of mosquito larvae (Buddington, 1941). An artificial media supplemented with autoclaved yeast has been used to rear normal adult mosquitoes (Lea et al., 1956; Akov, 1962).

Infusions of organic material have been used to successfully produce adult mosquitoes of many species (Atkin and Bacot, 1917; Barber, 1927; Hertig, 1936) in the ovaries and testis of *C. pipiens*. Brumpt (1938) described *Rickettsia culicis* Brumpt which had been fed 12 days previously on a subject with filariasis. Sellards and Sites (1928) frequently noted masses of rickettsiae in the lumen and epithelial cells of the hind gut of *A. aegypti*. These rickettsiae were present in specimens known to be infected with dengue fever virus, whereas they were not observed in control mosquitoes. More recently, Venters et al., (1971) reported the presence of rickettsia-like organisms in the midgut cells of 25% of *An. stephensi* examined. Since they observed such organisms only in stomachs free of malarial oocysts, they considered mosquito susceptibility to malarial infection can be influenced by the presence or absence of this organism. Yen and Barr (1971a) considered that the cause of cytoplasmic incompatibility in *C. pipiens* was due to *Wolbachia*-like rickettsia which are strain specific and localized in the follicles of the eggs. These microorganisms, especially abundant near the micropyle of recently laid eggs, lie in close proximity to sperm cells entering the micropyle in their passage to the female nucleus (Yen and Barr, 1971b). These workers suggest a deleterious effect by these microorganisms on presumably "incompatible" sperms so that they are unable to participate with the oocyte nucleus in the formation of a zygote. Rickettsia-like organisms are not novel to blood-sucking arthropods, however, and new examples continue

to be reported in such insects as *Rhipicephalus bursa* Canestrini and Fanzango (Friedhoff, 1970).

The bacteria associated with mosquitoes have only begun to be documented. Star and Micks (1957) (quoted by Ferguson and Micks, 1961) isolated 22 different bacteria cultures from 3 to 5 intact adult females of *C. fatigans* and 35 different strains from a homogenate of 49 specimens. Eighty percent of these were Gram negative rods.

Hinman (1932a) found bacteria as well as yeast within the eggs of *A. aegypti* and Kellen and Wills (1962) reported evidence of transovarial transmission of *Thelohania* sp. in various species of California mosquitoes. Chao et al. (1963), however, reported their failure to isolate microorganisms from within the eggs of 2 species of *Culex*, 2 of *Anopheles* and 2 of *Aedes*. Many workers have since reported the successful axenic rearing of adult mosquitoes from surface-sterilized eggs (Dougherty, 1959; Akov, 1964; Nayer, 1966; Wallis and Lite, 1970; Rosales-Ronquillo et al., 1972, 1973).

Chao and Wistreich (1959) isolated bacteria from the midguts of 46 adult *Culex tarsalis* Coquillett selected from a colony fed on apple slices for at least 2 days after emergence. Results of these isolations showed the presence of *Alcaligenes gutatus* (Zimmerman). *Achromobacter* ap., and *Aerobacter cloacae* Jordon, *Escherichia intermedia* Workman and Gillen, *Flavobacterium* sp., *Micrococcus* sp., *Micrococcus virians* Migula, *Proteus* sp., and *Pseudomonas* sp. Cultures of unidentified Gram-negative rods as well as *Saccharomyces* sp. and *Geotrichum* sp. were also reported. Chao and Wistreich (1959) also noted a higher ratio of midgut sterility in males than in females, and reported that males, after a forced blood meal could not digest the blood and died shortly after feeding. Wistreich and Chao (1960) isolated bacteria from midgut sections of 14 fourth-instar larvae of *C. tarsalis* that had been

raised in fresh tapwater and fed brewers yeast. Results of these isolations showed the presence of *Achromobacter* sp., *Lactobacillus* sp., *Micrococcus candidus* Cohn, *Micrococcus* sp., *Proteus rettgeri* Hadley et al., and *Pseudomonas* sp. Again, cultures of unidentified Gram negative rods and *Saccharomyces* sp. and *Geotrichum* sp. were observed. Isolations from the larval rearing water showed the presence of *A. cloacae*, *Klebsiella* sp., *Saccharomycetes* sp. and *Geotrichim* sp. Fourth instar larvae were found to contain examples of all the genera of bacteria isolated from adult *C. tarsalis* with the exception of *Aerobacter* sp., *Escherichia* sp., and *Flavobacterium* sp., whereas isolations from adults did not show the presence of *Lactobacillus* sp. or the coral pigmented form of *Micrococcus* sp.

Bacterial isolates from the midguts of adult *C. quinquefasciatus* fed apple slices for 2 days after emergence showed the presence of *Achromobacter* sp., *Flavobacterium* sp., *Klebsiella* sp., *Micrococcus caseolyticus* Evans, and *Pseudomonas* sp. (Chao and Wistreich, 1960). Midgut sections from 11 fourth instar larvae of the same species produced only *Bacillus* sp. and the yeast *Saccharomyces* sp. No apparent difference in the microbial flora from male and female adults were noted, with the exception of *Micrococcus* sp. and *Saccharomyces* sp. which were isolated from female mosquitoes only.

Wistreich and Chao (1961) extended their research to microbial isolation from larvae and adult *Aedes sierrensis* Ludlow and *Anopheles albimanus* Weidemann fed on a larval diet of yeast and adult diet of apple slices. Larval isolation from *A. albimanus* Weidemann fed on a larval diet of yeast and adult diet of apple slices. Larval isolation from *A. albimanus* showed the presence of *Alcaligenes* sp., *Bacillus* and unidentified Gram-negative rods. Adult *A. albimanus* showed *Achromobacter* sp., *Alcaligenes* sp., and *Corynebacterium* sp. *Saccharomyces* sp. was isolated from both larvae and adults.

A. sierrensis larvae contained *Bacillus circulans* Jordan, *Paracolobactrum intermedium* Borman and Prettger. *Achromobacter* sp. and *E. intermedia* were found in adults. *Saccharomyces* sp. were isolated from the larvae. Wistreich and Chao (1961) also reported that microorganisms isolated from the adult and larval environment were distinctly different from isolates of the midguts.

Wistreich and Chao (1963) reported microbial isolations from larvae showed the presence of the following bacteria: *Alcaligenes* sp., *Corynebacterium* sp., *Escherichia freundii* (Braak), *Kurthia bessoni* Hauduroy et al., *Sarcina flava* de Bary, as well as fungi belonging to the generas *Geotrichum*, *Penicillium*, and *Saccharomyces*. Isolations from adult mosquitoes showed the presence of *Achromobacter* sp., *Alcaligenes* sp., and *Saccaromyces* sp. Furthermore, they reported an especially high rate of midgut sterility in the adults of this species. Microorganisms were isolated from 5 to 21 females and 4 of 9 males. *Alcaligenes* sp. were found only in adult females.

Ferguson and Micks (1961a) instituted a similar series of experiments using adult female *C. fatigans* from a colony that had been denied access to food. By means of a sterile micro-dissection technique, they demonstrated the presence of *Lactobacillus* sp., *Alcaligenes* sp., *Pseudomonas* sp., and 2 unidentified cultures of Gram-negative rods. Bacterial isolates from the midgut of similarly treated adult female *An. quadrimaculatus* proved to be 2 strains of *Streptococcus* sp. and a species of *Aerobacter* (Micks and Ferguson, 1963). *A. aegypti* produced only a species of *Corynebacterium* and no microorganisms could be cultured from the midguts of adult female *Culex molestus* Forskal.

The midguts of newly emerged adults of *C. fatigans*, *C. molestus*, *An. quadrimaculatus*, and *A. aegypti* which were not allowed access to either

blood or sugar were examined by electron microscopy (Micks et al., 1961a). Ultrathin sections of *C. fatigans* midgut revealed the presence of intracellular microorganisms which Micks et al. (1961a) described as Gram-negative rickettsiae. Since these organisms did not appear to produce pathologic changes within the cells and were occasionally found in mycetome-like bodies. Micks et al. (1961a) suggested they might be symbiotes.

The question of a symbiotic relationship between mosquitoes and microorganisms has yet to be satisfactorily answered. Brooks (1965) generalized that arthropods possess symbiotic microorganisms only if they feed on nutritionally inadequate (i.e., incomplete) diets during their entire life cycle. Blood or serum is considered inadequate since it is deficient in B vitamins. Furthermore, since mosquitoes undergo complete metamorphosis from a larval stage spent as an omnivorous or scavenger-feeder, they are considered to pick up an adequate amount of vitamins and intestinal microflora (Brooks, 1964) in that stage to suffice for their entire life. Blood-feeders with incomplete metamorphosis which feed on blood during all immature stages, do have symbiotic microorganisms present, and as a rule, their alimentary tracts are otherwise sterile. Such arthropods are represented by bed bugs, sucking and chewing lice, and ticks and mites. Removal of symbiotes by axenic rearing procedures causes severe growth impairment, body malformations, lack of reproduction and death (Baines, 1956). Wigglesworth (1929) considered that symbionts provide some accessory food sutstances to tsetse flies that are similar to the vitamins necessary for mammalian growth. B vitamin supplementation of the blood meal fed to axenic blood-feeders does, in fact, alleviate the growth factor deficiency but fails to restore normal reproduction (Pucha, 1955). Aschner (1934) found that sumbionts are a source of food for the body louse *Pediculus*

hominus hominus Linnaeus. Young lice died within 5 or 6 days when deprived of the mycetomes that contain symbionts. *Actinomyces rhodnii* Erickson in the gut of *Rhodnius* sp. are essential for the maturation of the insect (Brechner and Wigglesworth, 1944).

In order to obtain further evidence of a possible symbiotic relationship between *C. fatigans* and its associated microorganisms, Micks and Ferguson (1961b) placed a number of females on a chloroamphenicol-dihydrostreptomycin-sugar solution diet for periods of 5 to 12 days. Mosquitoes relieved of their microorganisms by such means were subsequently allowed to take a blood meal from canaries infected with *Plasmodium relictum* Grassi and Feletti to ascertain possible effects on host immunity. Antibiotic treated females became inactive, lacked coordination, were unable to fly, and when dissected, their midguts were found to contain large masses of undigested blood as long as 10 days after the blood meal. The normal rate of digestion of human blood, although variable with the species and day-length, is reported to be 31 to 48 hours (O'Gower, 1956). Eighty-five percent of the treated mosquities and 74% of the untreated controls were infected with *P. relictum*. In general, antibiotic treated individuals contained about twice the number of oocysts per midgut as did controls.

Micks and Ferguson (1961b) concluded that microorganisms play a significant role in the susceptibility or resistance of the mosquito to the malarial parasite. They mention the possibility of competition between the microorganisms and the malarial parasites for essential nutrients, in which case, the antibiotic would work in favor of the *Plasmodium*. Alternatively, they suggested that certain strains of these microorganisms could supply factors needed by the host for metabolic processes and defense mechanisms, and that killing the organisms increases host susceptibility to malaria.

Unfortunately, their work appears to have been discontinued at this stage. Furthermore, the feeding of antibiotic diets, besides killing most, but not all, internal microorganisms, may have produced additional effects on host physiology not considered by these workers.

Shymala et al. (1960) reported that chloromycetin in the diet of the silkworm influenced the digestion and utilization of protein, fat, and minerals. Tetracycline, oxytetracycline, and chlorotetracycline have been shown to combine specifically with the mitochondria of living monkey kidney cells and with the cells of *Salmonella typhosa* White (Dubuy and Showacre, 1961). Although the mammalian cells remained alive for days, there is much that remains unexplained about the effects on metabolism and nutritional requirements of antibiotic-treated cells.

The roles of microbiotic symbiotes within mosquito midguts are manifold. Hinson (1933) found within the bacterial cell, a factor which stimulated growth of mosquito larvae. Rozebloom (1935) considered that different species of bacteria possessed varying nutritional properties for mosquitoes. *E. coli, B. subtilis, B. mycoides* and *P. florescens* were equally satisfactory as a sole source, while *S. lutea* and *Pseudomonas pycanea* Migula (*P. aeruginosa* Schroeter) were detrimental. Arnal (1950) considered that specific symbiotes were essential for the digestion of erythrocytes in mosquitoes and described intracellular organisms in midguts of *C. pipiens* which secreted enzymes capable of hemolyzing erythrocytes. Micks and Ferguson (1961b) found undigested blood only in those mosquitoes in which the microbial flora had been reduced or eliminated by the antibiotic diet. Ferguson and Micks (1961b) also noted that one of the bacterial strains isolated from the midgut of *C. fatigans* produced hemolysis of human blood. The work of Chao and Wistreich (1959) support this relationship between midgut microorganisms and the proper

digestion of blood. They found a higher ratio of midgut sterility in males of *C. tarsalis* and also noted that males could not digest blood with which they had been fed. Terzian et al. (1952) reproduced the "indigestion" syndrome in *A. aegypti* by feeding calcium and calcium-antibiotic combinations before and after engorging. Only digestion of the hemoglobin fraction of the blood was inhibited. The effect of the antibiotic (they reported) was to potentiate the inhibitory effect of the cations upon the particular enzyme system involved. Terzian (1953) also reported that streptomycin and chloromycetin increased susceptibility of *A. aegypti* to *Plasmodium gallinaceum* (Brumpt). The author apparently did not consider that this increase in susceptibility might be due to antibiotic mediated alteration in host microbial flora.

Statement of the Problem

This dissertation is a series of 4 investigations on the relationship between filarial vector and its resident microflora in the midgut. The participation of these microflora in the transmission of parasitic disease is of special interest. The *a priori* premise is that mosquitoes acquire their life-long complement of midgut bacteria as indiscriminate filter-feeding larvae. During growth, competition among microorganisms establishes a microecosystem of bacterial types which are retained throughout life. The arthropod midgut is the only part of the alimentary tract which is not shed during molting. This bacterial complement is individually distinct because of its fortuitious acquisition, yet species specific because of the consistencies of egg-laying site selection among mosquito species.

The influence of this internal microhabitat upon potential parasites passing through the alimentary tract may be manifold. It may be of sufficient importance ot determine the fate of the pathogen, and thus, the efficiency of the vector. The recently developed techniques in gnotobiology presented the opportunity of selectively separating the arthropod vector from its indigenious microflora.

The first problem was to titrate levels of filaria infection in the mosquito, determine their effect on the mosquito, and the ultimate fate of the larvae. Of parallel interest was the phenomenon of early death in infected mosquitoes. This investigation is reported in Results, I.

Secondly, a system had to be devised whereby a very efficient filaria

vector such as *An. quadrimaculatus* could be raised, maintained, and infected under gnotobiotic conditions. Previously reported techniques were not appropriate to this species. Results, Section II describes this effort.

The problem of defining the "normal" bacteriologic flora of mosquitoes was also unique. No reports of bacteriological evaluations of wild-caught mosquitoes have appeared in the literature. Furthermore, such reports as have appeared, were not quantitative. Results III reports a quantitative and qualitative evaluation of 5 groups of wild and colonial mosquitoes.

Finally, the effect of the normal flora on the establishment of filarial larvae in a susceptible mosquito is investigated. Pure cultures of bacteria isolated from wild mosquitoes are "added back" into gnotobiotic mosquitoes as monocontaminants. These mosquitoes, as well as conventional and bacteria-free specimens were infected with *D. immitis* larvae. Forty-eight hours later, all mosquitoes were terminated and the degree of filarial development assessed. This is reported in Results, Section IV.

RESULTS

I. BIOASSAY OF EARLY VECTOR MORTALITY FOLLOWING *DIROFILARIA IMMITIS* LEIDY INFECTION

Introduction

The most salient problem attending experimental attempts to transmit dirofilariasis through laboratory mosquitoes is the initial high vector mortality. Various authors including Bradley (1953a), Kershaw, Lavoipierre and Chalmers (1953), Webber and Hawkins (1953), and Weiner and Bradley (1970), have reported mosquito mortality approaching 100% in the first days following the blood meal. A common antecedent to death appears to be distension of the abdomen persisting 2 to 3 days, which Weiner and Bradley (1970) interpret as a result of improper digestion of blood.

Mortality subsequent to the ingestion of large numbers of infective larvae in the blood meal has been shown to be a result of mechanical destruction in the insect tissue (Pistey, 1959; Villavaso and Steelman, 1970). Larval destruction of the Malpighian tubules as they leave these organs on the fifteenth day is an obvious cause of mortality. Esslinger (1962) documented three pathological stages consequent to infection of *Anopheles quadrimaculatus* Say with *Brugia pahangi* Buckley. These include perforation of the peritrophic membrane, destruction of the gut epithelial cells and puncture of the basement membrane, and finally, destruction of the fat body. However, death due to mechanical destruction after infestion of low or moderate numbers of microfloriae seems inappropriate in such successful filarial vectors as mosquitoes are known to be.

The incidence of mosquito mortality during the first 3 days after infection with *D. immitis* may be the result of (A) mechanical action of the larvae, (b) a toxic factor elaborated by the larvae, or in response to the larvae, or (c) disturbances in endocrine function occasioned by the larvae. Even subtle physiologic insults produced by filaria larvae may be greatly magnified in the female mosquito as all the endocrine machinery of egg-laying begins to function.

The following bioassay is an effort to determine which of these 3 possibilities, if any, is the cause of vector mortality in the first 3 days following infection with *D. immitis* larvae.

Materials and Methods

An. quadrimaculatus (Gainesville strain) were reared and maintained in an insectary at the Insects Affecting Man Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Gainesville. Ambient conditions were maintained at 26°C, 80% RH and 12 hr LD. Adult females were fed 5 days on a 2.5% sugar solution after emerging; starved for 2 days (Terzian et al., 1957) and fed on various preparations through either a capillary tube or a heated gutta percha membrane. Such preparations included live microfilariae, dead heat-killed microfilariae, dead homogenized microfilariae, and either the homogenized midguts, heads, or remainders of the bodies of previously infected female mosquitoes.

As mosquitoes completed feeding, they were transferred to holding cages in groups of 20 where mortality observations were made twice daily. Moribund mosquitoes were dissected and examined for the presence and condition of *D. immitis* larvae. Microfilariae were collected from filaremic dogs using the Klein and Bradley (1973) modification of the Knott technique.

The microfilariae were separated from the cellular components concentrated by centrifugation, adjusted to a 2,000 microfilariae per 0.5 ml concentration with normal saline (Wong, 1964). The cellular components were then reintroduced to bring the volume up to 1 ml. Homogenized material was sonicated in cold saline, brought up to 0.5 ml volume with saline, and up to 1 ml volume with canine red cells obtained from an uninfected donor dog.

Results

The mortality of mosquitoes taking blood meals with differential filaremias is shown in Table I-1. A concentration of 1,000 microfilariae per ml of the blood meal permits optimum mosquito survival and results in an average of 12 microfilariae per mosquito. Gross dissection of mosquitoes succumbing from this level of infection revealed no notable tissue destruction in the 3 day critical period. The Malpighian tubules contained active microfilariae in the cells at the distal end. The tubules were intact, and entirely normal in appearance. Digestion of infective blood meals was consistently retarded in comparison to digestion in control mosquitoes.

Table I-2 shows that no significant mortality was produced by feeding either dead intact microfilariae or sonicated microfilariae bodies to newly emerged female mosquitoes. This finding eliminates consideration of a toxic factor produced by, or within the bodies of microfilariae.

Table I-3 shows the effect of feeding the homogenized body parts from mosquitoes fed 24 hours previously on infective blood meals. Neither midgut preparations, head preparations, nor remaining body parts contain factors which duplicate the high vector mortality experienced when viable microfilariae are fed.

Table I-1 Effects of Differential Filaremia on Mortality of *Anopheles quadrimaculatus* SAY

Concentration Larvae/ml	Number Females Feeding	Cumulative Mortality in 3 days	Percent Mortality
0	39	3	8
1,000	40	10	25
5,200	40	18	45
9,600	40	10	25
10,000	40	20	50
20,000	36	8	22
29,000	40	36	90
30,000	40	38	95
66,000	36	34	94

Table I-2 Effects of Feeding Dead Intact, and Homogenized Microfilaria to Female *Anopheles quadrimaculatus* SAY

Dead Intact Larva (1,000 Larvae/ml)	Number Females Feeding	Cumulative Mortality in 3 Days (%)
Trial 1	24	0
2	23	2
3	20	0
4	20	0
Sonicated Larvae (1,300 Larvae/ml)		
Trial 1	30	1
2	30	2

Table I-3 Effects of Feeding Homogenized Body Parts From Females Fed Infective Meals 24 Hours Previously

Adjunct to Blood Meal	Number Females Feeding	Cumulative Mortality in 3 Days (%)
Homogenized Midgut	58	5.1
Homogenized Remainder of Body	73	4.1
Homogenized Heads	20	0

Discussion

Results of this study show that living microfilariae produce at least 25% mortality in infected *An. quadrimaculatus* during the critical first 3 days post infection. This mortality could be the result of either occult destruction of mosquito tissue, or some factor produced by, or in response to the microfilariae. Dead, intact larvae do not stimulate a comparable mortality reinforcing the explanation of mechanical destruction. When suspensions of dead homogenized microfilariae were fed, a comparable level of mortality was not achieved, indicating that a lethal factor could still be intrinsic t the mosquito. Feeding homogenized midguts of previously infected mosquitoes failed to reproduce mortality suggesting the female endocrine system as a possible source of the factor.

Blood feeding in non-autogenous female mosquitoes initiates a neuroendocrine arc involving endocrines from the brain which stimulate the ovaries to proceed with egg formation (Larsen, 1958). Neither head preparations, nor whole-body homogenates from previously fed mosquitoes contained factors which reproduced the high mortality in newly emerged mosquitoes. An endocrine related factor is not supported by these findings.

Developmental studies by Kartman (1953b) and Pistey (1957) have shown that prelarvae migrate to the Malpighian tubules very soon after ingestion, and Pistey (1959) infers that the sudden invation of the tubules is selectivity fatal. Pistey (1959) reports that the most critical period in *Dirofilaria tenuis* Chandler infected mosquitoes was the initial 3 days when microfilariae enter the Malpighian tubules. Intermill (1973), however, describes the migration of *D. immitis* larvae to the Malpighian tubules of *Aedes triseriatus* Say and notes that it proceeds without any apparent

histological damage to the gut or tubules.

Inasmuch as no obvious destruction of the Malpighian tubules was apparent in the present study by gross dissection, and no toxic factors could be demonstrated, the cause of vector mortality remains unexplained. Viable, pre-larvae of *D. immitis*, however, are extremely active and subtle alterations in Malpighian tubule function could be produced by their presence with little physical evidence. The destruction of tubule cell cytoplasm and cellular membranes by invading larvae has been known since the earliest authors (Noe, 1901). Subtle destruction of the cellular components of the tubules may impair nitrogenous excretion, or the retention or reabsorption of dietary components necessary for life. Even a minute perforation of the tubule integrity could allow leakage of uric acid and other nitrogenous constituents into the haemocoel with terminal results for the mosquito.

It would seem appropriate to implicate "indigestion" as a factor in the early death of filarial vectors. Lewis (1952) noted that the presence of microfilaria of *Onchocera volvulus* Leuchart interfered with the formation of the peritrophic membrane of *Simulium damnosum* Theobald and Wanson (1950) reported that blood contained in the stomach heavily infected *Similium* which died a few days after an infective meal appeared to be undigested. Similar observations on other filaria - host combinations were reported by Roubaud et al. (1936) and MacKerras (1953). Lavoipierre (1958) concluded that the most likely cause of vector death was extensive damage to the peritrophic membrane rendering digestion almost impossible. The peritrophic membrane was impossible to demonstrate in dying, heavily infected flies with undigested blood meals, whereas the membrane was present in controls in which the blood meal was in an advanced state of digestion.

RESULTS

II. AN INTEGRATED SYSTEM FOR THE PRODUCTION OF GNOTOBIOTIC *Anopheles quadrimaculatus* SAY

Introduction

A host of investigators beginning with Schottelius in 1899 have demonstrated the fallacy of Pasteur's assumption that the host-microflora relationship is obligate. Since then, the germ-free animal has given the biologist one of his most valuable models to study the interactions of host and microorganisms.

The development of gnotobiotic arthropods was initiated by Trager (1935a, b, 1936, 1937) who reported the growth of larval *Aedes aegypti* (L) in sterile media containing essential nutritional compounds. In the 4 decades subsequent to that, the dietary requirements of *A. aegypti* in axenic culture have been thoroughly studied by a number of workers (Goldberg and DeMeillon, 1948a, b; Lea et al., 1956; Lea and DeLong, 1958; Singh and Brown, 1957; Akov, 1962; Lang et al., 1972). However, only a few species other than *A. aegypti* have been successfully cultured in sterile media. They include: *Culiseta incidens* (Thom) (Frost et al., 1936), *Culex pipiens pipiens* (L) (Buddington, 1941; Dadd et al., 1973), *Culex molestus* Forskal (Leichenstein, 1948), *Aedea taeniorhynchus* (Wiedeman) (Nayar, 1966), *Culex salinarius* Coquillett (Wallis and Lite, 1970), and *Anopheles stephensi* Liston (Rosales-Ronquillo et al., 1973).

The objective of most of these studies has been to define the nutritional requirements of mosquitoes, rather than to investigate the microflora-host relationship. Of equal interest is the role of bacteria in the biology

of the host and in the pathogenesis of certain disease mechanisms.

The public health significance of the mosquito makes it one of the most appropriate arthropods to receive such attention. *Anopheles quadrimaculatus* Say is a major vector of canine heartworm disease in the eastern United States. This paper reports the first successful rearing of *An. quadrimaculatus* under bacteria-free conditions and the successful use of a lucite capsule specifically designed for gnotobiotic studies with blood-feeding arthropods.

Materials and Methods

Egg sterilization method

An. quadrimaculatus eggs were collected from colonies maintained by standard procedures (Anon., 1973) at the USDA Agricultural Research Service, Insects Affecting Man Laboratory in Gainesville, Florida. Egg sterilization was accomplished in 3 cm long cylinders made from the truncated upper half of a disposable plastic syringe barrel. Organdy netting was cemented over the lower end to form a deep non-wettable dipping cylinder for egg handling. These cylinders fit neatly through the neck of 25 mm x 10 mm screw cap tubes and were autoclaved as a unit before use. With several hundred eggs in the cylinder, the cylinder was dipped in the first tube containing 70% ethyl alcohol for 15 seconds to facilitate wetting the egg surface (Hinton, 1968). The cylinder was then transferred to a tube containing 10% Zephiran chloride[1] for 15 minutes with occasional agitation. [White's solution (White, 1931) for 15 minutes is also an effective germicide.] Eggs usually sank to the bottom of the cylinder during immersion in the germicide. The cylinder was next transferred through 2 tubes of sterile water for 5 minutes each. An aliquot

[1]Winthrop Laboratories, New York, N.Y.

broth. The culture flasks were incubated with a 12-hour light cycle in an incubator. Pupation began at the 8th day, and female pupa, recognized by their larger head, were withdrawn with a syringe fitted to a long 14 g hypodermic needle.

The gnotobiotic arthropod module

The gnotobiotic arthropod module or GAM (Figure II-1) is a sealed lucite capsule allowing sugar feeding at one end, and blood feeding through a heated artificial membrane at the other. Mosquito pupae were injected through the rubber stopper (#3) with a small amount of water, which allowed adult emergence and humidified the capsule when held at 27°C.

The GAM was constructed from .3 cm lucite cylinders measuring 7.5 cm i.d. x 10 cm and cemented with PVC industrial solvent. About 2/3 of the inner surface of the cylinder was covered with plastic screen to provide resting sites. A Gelman[3] filter support (#5,9) was bonded to the GAM floor (#8) and interposed a stretched gutta percha membrane (#6). The outer collar (#9) of this filter support was threaded so it can be inverted and screwed tightly into the floor (#10) of the heating manifold. A teflon[R] seal (#7) insured watertight integrity. The GAM was sterilized with the membrane in place in an ethylene oxide chamber, or autoclaved if the membrane was to be inserted later (30 minutes at 15 lbs and 120°C).

Figure II-2 shows a battery of 4 GAMs in the blood feeding mode in place in the heating manifold. The blood meal was injected through the rubber stopper of the blood tube (#11) and was kept at a constant temperature of 32°C. Heated water was pumped from a laboratory water bath into the manifold inlet (#3) causing an overflow (#2) return to the water bath.

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[2] Nutritional Biochemicals, Cleveland, Ohio

[3] Gelman Instrument Co., Ann Arbor, Mich.

of 25-50 eggs was taken up in a small amount of water in a sterile pipette and transferred into a sterile dry tube containing 1.5 cm x 10 cm strip of filter paper. The eggs were dispersed over the strip in an amount of water just sufficient to thoroughly wet the paper strip and the tubes were incubated on their sides at 27°C. Eggs hatched in 24 hours and the larvae were seen crawling on the surface of the paper. Within the next 3 hours, the tubes were filled with sterile water, causing the empty egg shells to sink, and the sterile larvae to float to the surface for recovery.

The larval diet

Formulation of the larval diet met the following requirements: good growth and adult emergence after autoclave sterilization, standardization, and ease of preparation. The composition of this diet is illustrated in Table II-1. The RNA component and the casein were dissolved in 250 ml of water over heat. Dilute KOH was added to maintain pH at about 7.0 as solution proceeds (Dadd et al., 1973). The hog supplement[1] was previously prepared by drying at 140°F for 1 hour, ground in a mill, and sieved twice through a 50 mesh sieve. The remaining ingredients were added and final volume brought up to 1 liter. The pH was adjusted to 7.0 with KOH and dispensed to flat-sided 16 ounce screw cap prescription bottles in volumes sufficient to produce a depth of .5 cm (about 50 mls). Each culture bottle contained a 5 cm x 15 cm strip of plastic screen material for emerging adults to rest on when cultures were continued to that stage. Finally, the culture bottles were autoclaved for 15 minutes.

Newly-hatched larvae were inoculated 1 larva per 2 ml of media using a sterile Pasteur pipette. Sterility was monitored by inoculating an appropriate larval sample into tubes of thioglycollate[2] broth and nutrient[2]

[1] Ralston Purina, St. Louis, Missouri
[2] Difco Laboratories, Detroit, Michigan

Figure II-1. Gnotobiotic Arthropod Module (GAM) used for the Maintenance and Blood Feeding of *Anopheles quadrimaculatus*: 1. Sugar delivery tube and wick, 2. Cylinder top, 3. Rubber stoppers, 4. Lucite cylinder, 5. Outer collar of Gelman filter support, 6. Gutta percha membrane, 7. Teflon seal, 8. Cylinder floor, 9. Inner Gelman filter support, 10. Floor of heating manifold, 11. Blood delivery tube.

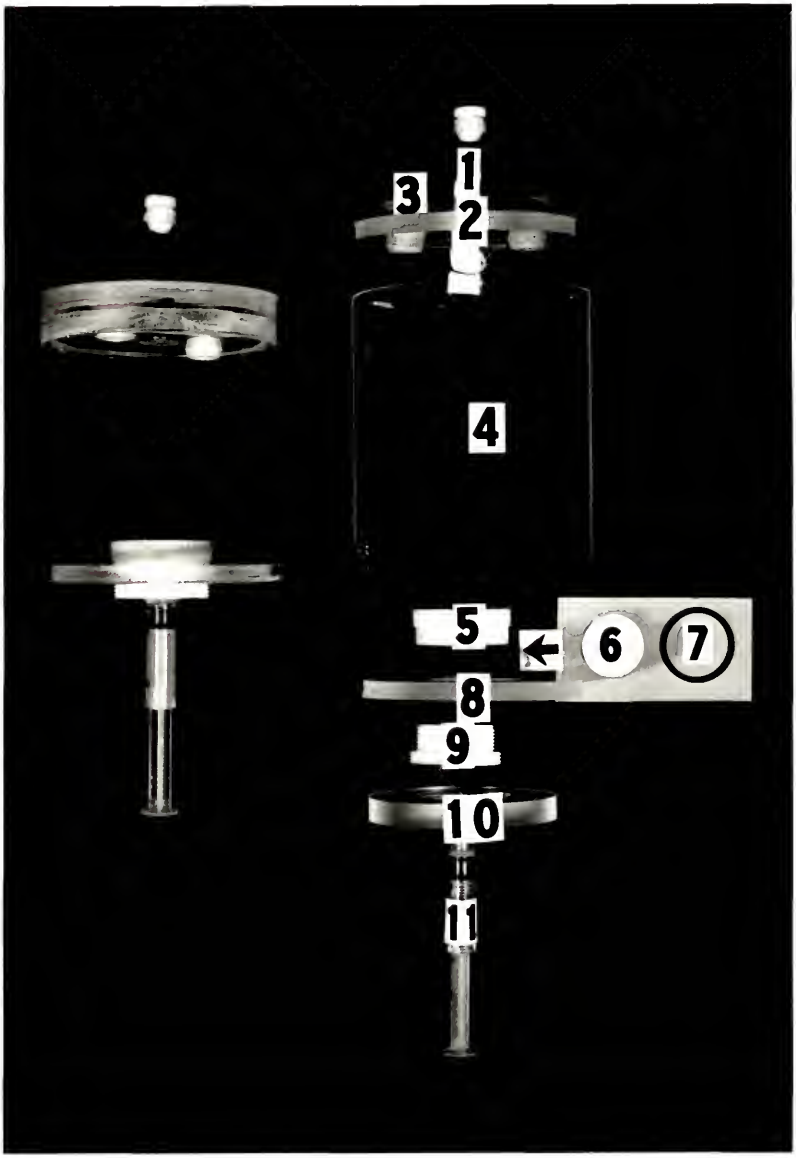
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Figure II-2. Battery of 4 GAMs in Position for Blood Feeding in Heating Manifold: 1. Top of manifold, 2. Overflow into waterbath, 3. Inlet from waterbath, 4. Manifold tank.

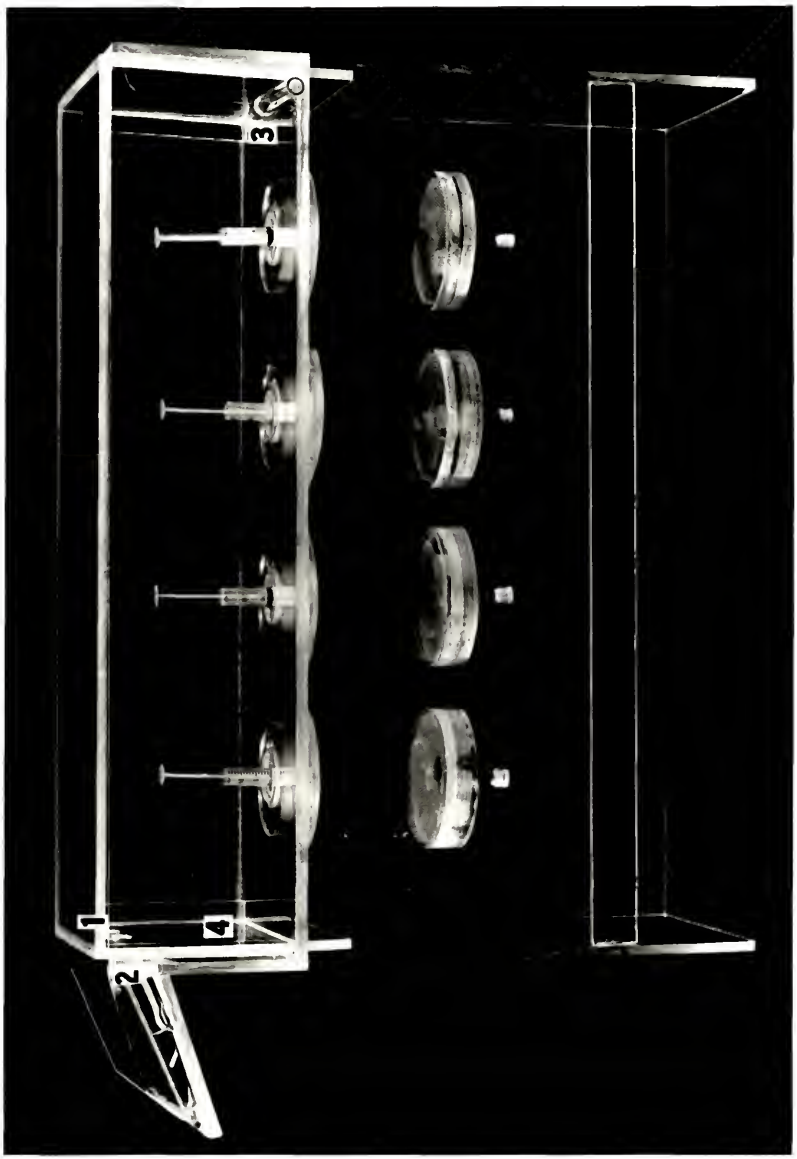
1
2
3
4

Table II-1 Composition of Diet for Rearing *Anopheles quadrimaculatus* Say

Ingredient	mg/1,000 ml
Purina S.E. Hog Supplement[1] 40%	1,000
NBC Liver Powder[2]	500
Casein[2]	50
Brewer's Yeast[2]	500
Ribonucleic Acid[2]	1,000
Vitamin Diet Fortification Mix[2]	1,000
Salt Mixture - W (Wesson)[2]	250

[1]Ralston Purina Co., St. Louis, Missouri

[2]Nutritional Biochemicals Corp., Cleveland, Ohio

Survival of mosquitoes in the GAM was quite good provided leakage of blood and sugar solution was prevented. Normally, 12-15 adult mosquitoes were maintained in each GAM.

Results

Egg-sterilization method

Several methods for the surface sterilization of mosquito eggs were tried including peracetic acid (Doll et al., 1963), antibiotics (Epps et al., 1950), Lysol[R] (Adkin and Bacot, 1917), Clorox[R] (Lea, 1957), iodine, alcohol, Zephiran[R] (Dadd et al., 1973) and White's solution (White, 1931). Only the latter 2 germicides consistently produced viable sterile *An. quadrimaculatus* larvae, and then, only when the eggs were held on moist filter paper. Effective egg sterilization deprived the egg of its ability to float, and eggs which sink are non-viable. An extended embryonation period is another result of several of the germicides tested. Bacteria-free, viable larvae could be consistently produced in 24 hours, using the sterilization procedure described.

The larval diet

The larval diet was a composite of the diets suggested by several workers. Liver powder[1] and brewer's yeast[1] are traditional protein sources (Trager, 1935) which, in the present study, were included as first food for first instar larvae. Purina S.E. hog supplement 40%[2] is an excellent protein source for older larvae and was used as the standard diet for conventional *Anopheles* sp. Dadd et al. (1973) reported that ribonucleic acid, and soluble casein, because it contained an essential sterol as a contaminant,

[1] Difco Laboratories, Detroit, Michigan

[2] Ralston-Purina, St. Louis, Missouri

were both critical components of their mosquito diet. For that reason, both were included in the present diet.

The 8 vitamins essential to growth and development of mosquitoes (Akov, 1962) were more than supplied by the addition of 1 gm/liter NBC[1] Vitamin Fortification Mixture. Although probably not utilized (Clements, 1963), it also contains vitamins A, B_{12}, C. D, and E. Furthermore, it is compounded in dextrose which, as Nayar (1966) and Johnson (1969) found, contributed to maximum adult survival when included in *Aedes* sp. diets. Originally, this vitamin component was sterilized by Millipore filtration,[2] but like Dadd et al. (1973), no adverse effect could be detected when the vitamin component was autoclaved along with the rest of the media. Use of the Salt Mixture-W[2] very conveniently approximated Trager's (1935b) salt mixture and evidently provides excellent buffering capacity in this application.

Immoderately high osmotic pressure of the culture media can be responsible for poor growth and development on otherwise adequate chemically defined media (Nayar, 1966). *An. quadrimaculatus* larvae are highly sensitive to high osmotic pressure which can cause larval death in the first 25 hours of culture. For that reason, the diet described here was very dilute in comparison to other diets (Rosales-Ronquillo et al., 1973).

Developmental time of germ-free mosquitoes was approximately the same as for conventionally-reared mosquitoes. Approximately 75% of the first stage larvae survived to the pupal stage on the 8th day. No reduction of vigor in pupae or adults was noted.

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[1] Nutritional Biochemicals, Cleveland, Ohio
[2] Millipore Corporation, Bedford, Mass.

Use of the Gnotobiotic Arthropod Module

The GAM provided an air-tight sterilizable capsule to maintain 12-15 gnotobiotic arthropods. Some practice was required in determining how much water is injected with the pupae. In general, as little as possible will suffice to humidify the capsule but will not entrap adults. Leakage of sugar solution from the wick and blood seepage around the membrane are other problems which may be encountered. Both can entrap mosquitoes if allowed to develop.

Discussion

Considerable flexibility is possible with the GAM and it should provide appropriate experimental units for a variety of blood feeding arthropods. The GAM may be successfully employed in comparative nutritional studies of related arthropods, the identification of growth factors, trace elements, and the effects of environmental variations. The influence of contaminating microorganisms may be selectively eliminated for the study of host metabolism, growth and aging under a variety of conditions.

Insect pathogen studies would be an appropriate application of the GAM. Pathological material may be safely isolated, and its effects evaluated without the influence of bacterial contamination. Pathological effects of insect microsporidian parasites may be evaluated in the sterile intestine and in conjunction with various combinations of indigenous microflora.

The GAM may have wide application in basic research on the relationship between an arthropod host and the parasites it vectors. Previous gnotobiotic studies have shown that the success of many parasitic diseases depends not only on the etiological agent, but also on the host's intestinal microflora (Phillips and Gorstein, 1966; Micks and Ferguson, 1961;

Bradley et al., 1964). Parasites and potential parasites passing through the arthropod host's alimentary tract are exposed to both the microorganisms that reside there, and to the intestinal climate which may, in part, be influenced by them. Bacteria present at the host-parasite interface may influence the ability or disability of the arthropod to vector parasitic disease.

RESULTS

III. THE NATIVE MIDGUT BACTERIAL FLORA OF SEVERAL WILD AND COLONIZED MOSQUITO SPECIES

Introduction

The ability of mosquitoes to vector disease is of such significance to world-wide public health, that a better understanding of their biological relationships with microorganisms in general is critical. The intimacy existing between the host and its midgut population of microorganisms suggests more than chance environmental contamination. Instead, years of conjunct evolution may have produced a highly integrated gastrointestinal ecosystem which may ultimately participate in the capacity of an insect to transmit disease.

On the basis of a long series of comparative studies of albino mice from different colonies, Dubos et al. (1965) have concluded that the indigenous (or normal) microbiota of these animals exerts a profound influence on their rate of growth, their efficiency in the utilization of food, and their resistance to infection, toxic substances, and other stressful agencies. Many attributes of mice which are characteristic of the colonies from which the animals were derived are in reality determined not by genetic endowment, but by the microbiota prevailing in the colony. There is no reason to suppose that insects should be unique among animals in their association with microorganisms.

In the course of another study, it became of interest to define the midgut bacterial population of wild filaria vectors. Two pairs of inde-

pendent workers (Chao and Wistreich, 1959, 1960; Wistreich and Chao, 1960, 1961; Ferguson and Micks, 1961; Micks et al., 1961; Micks and Ferguson, 1961, 1963), have produced a series of reports on colonized mosquitoes which indicate that the flora of adults is different from that of larvae; it varies among the different species, and it is distinct from bacteria in the specimen's environment. Although there was always a high percentage of sterile individuals, the microorganisms recovered from contaminated specimens are generally non-pathogens widely distributed in food, water, soil, and intestines of animals.

These reports, however, were qualitative evaluations of laboratory insects maintained under standard conditions in established colonies. No reports have appeared which define the microbial composition of wild naturally-feeding mosquitoes. The following report is a qualitative and quantitative evaluation of several hundred adult specimens of colonized *Anopheles quadrimaculatus* Say, wild *An. quadrimaculatus*, wild *Anopheles crucians* Wiedemann, wild *Aedes infirmatus* Dyar and Knab, and *A. crucians* complex, trapped at 6 stations in Gainesville, Florida.

Materials and Methods

One-hundred adult female specimens of *An. quadrimaculatus* (Gainesville strain) were selected over a period of time from the colonies at the USDA's Agricultural Research Service, Insects Affecting Man Laboratory in Gainesville, Florida. They had been fed a liver[1]:yeast[1] mixture

[1] Nutritional Biochemicals, Cleveland, Ohio

for the first 3 larval days and ground S.E. Hog Supplement[1] for the balance of the larval period. Adults were held 5-7 days after emergence, fed a 2.5% sugar solution, and starved 24 hours prior to dissection.

Several hundred wild mosquitoes were trapped from 6 field stations around Gainesville, Florida, with a CDC[2] type light trap using dry ice as a carbon dioxide source. The species composition of the wild females selected for study was: *An. quadrimaculatus*, 25 specimens; *An. crucians*, 80 specimens; *A. infirmatus*, 12 specimens; *An. crucians* complex, 20 specimens. These mosquitoes were also held 24 hours without food before dissection.

Both oral and anal openings of each insect were sealed with Duco[R] cement by bonding the insect at these points to a plastic cover slip. Seepage of the germicide into the digestive tract was prevented, and the coverslip effectively anchored the insect for dissection beneath the surface of sterile water. The mounted insect was immersed in 70% ethanol for 30-60 seconds, rinsed and dissected under sterile water. A section of abdominal integument was removed and placed in a tube of thioglycollate broth[3] as a sterility check. Gentle distention of the lower abdomen exposed the digestive tract, and the midgut was retracted and removed without puncture. The midgut was then homogenized in .4 ml sterile water in a glass tissue homogenizer. One-tenth ml aliquots of the homogenate were then streaked for isolation onto duplicate Eugon[3] agar plates, 1 Eugon

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1 Ralston Purina Co., St. Louis, Mo.

2 National Communicable Disease Center, Atlanta, Ga.

3 Difco Laboratories, Detroit, Mich.

agar plate fortified with 5% defibrinated sheep blood, and 1 tube of fluid thioglycollate[1]. Incubation took place at 37°C, 25°C, 37°C, and 37°C, respectively. A total count of organisms present in the midgut was made by counting all of the colony-forming units, appearing in Eugon agar after 24 hours, and multiplying by a factor of 4. Each different type of colony appearing on the 3 agar plates was counted, sampled, assigned an isolate number and streaked for pure culture on Eugon [1] agar. When growth appeared in the thioglycollate[1] tube, inoculations were made into veal infusion agar tubes[1] to differentiate obligate anaerobes. Yeasts and fungi were inoculated into Sabouraud maltose agar[1].

When pure cultures were apparent, inoculations were made into triple sugar iron agar[1] (TSI) and brain-heart infusion agar[1] (BHI) slants which were maintained as stock cultures at 9°C. Colony morphology, chromogenesis, and Gram-reaction were determined at this time. Table 1 lists the procedure in the presumptive determination of bacterial genera. Hydrolytic abilities were determined in agar supplemented with 5% soluble starch, 5% gelatin, 5% Wesson oil, or 5% alphacel[1]. Oxygen relations were demonstrated in duplicate tubes (one overlaid with sterile mineral oil) of OF[1] media and incubated for 5 days. The presumptive identification of Gram-negative enteric bacteria as well as the catalase test were performed on TSI. Decarboxylase production was tested in lysine, arginine, ornithine, and control tubes. An I.M.V.i.C. series (indol production, methyl red pH, Voges-Proskauer, and citrate utilization) was performed in appropriate media. Both the nitrate reduction

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[1] Difco Laboratories, Detroit, Mich.

test and motility were determined in nitrate agar[1] slants. The type of milk reaction was demonstrated in litmus milk[1] media and the hydrolysis of urea was determined on urea agar[1]. Carbohydrate fermentations were demonstrated on cystine trypticase agar[1] plates overlaid with any 10 carbohydrate differentiation[1] discs listed in Table 1.

Data analysis was by means of a Statistical Analysis System (SAS) program written by Barr and Goodnight (1972). A frequency distribution of the species composition of midgut bacterial flora was computed. Correlation coefficients and probability statements were calculated on the frequency of occurrences of all pair combinations of bacterial isolates.

Results

Aedes infirmatus Dyar & Knab

The average midgut bacterial count of 8 specimens of *A. infirmatis* was 263. No bacteriologically sterile specimens were encountered in the sample. Table III-1 illustrates the species composition of midgut bacterial flora isolated from all 5 groups of mosquitoes. The most frequently isolated bacterial species in this mosquito group was *Bacillus cereus* Frankland and Frankland, non-pathogenic *Corynebacterium* sp., and *Micrococcus luteus* (Schroeter).

Anopheles quadrimaculatus Say (wild)

Sixty-one percent of the wild *An. quadrimaculatus* examined were found to be bacteriologically sterile, which was the highest rate of

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[1] Difco Laboratories, Detroit, Mich.

FIGURE III-1

Procedures in the Presumptive Determination of Bacterial Genera

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1. Hydrolysis Reactions: Starch Hydrolysis
   Gelatin Hydrolysis
   Lipid Hydrolysis
   Cellulose Hydrolysis
2. Oxidative Fermentation Reaction
3. Catalase Test
4. Triple Sugar Iron Slant
5. Growth Study: Blood Agar
   Eugon Agar @ 25°C
   Eugon Agar @ 37°C
6. Decarboxylase: Lysine
   Arginine
   Ornithine
   Control
7. I.M.V.i.C. Series
8. Motility
9. Nitrate Reduction Test
10. Litmus Milk Reaction
11. Urease
12. Fermentation Reactions (any 10):

Arabinose	Glucose	Maltose	Sorbitol
Cellobiose	Glycerol	Mannose	Sucrose
Dulcitol	Inositol	Mannitol	Trehalose
Fructose	Inulin	Raffinose	Xylose
Galatose	Lactose	Salicin	Control

TABLE III-1

Species Composition of Midgut Bacterial Flora in 5 Groups of Mosquitoes

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Chromatia* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus epidermidis*	*Aeromonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Micrococcus* sp.	*Achromobacter* sp.	*Pseudomonas quercifaciens*	*Nocardia* sp.	*Flavobacterium* [illegible]	*Arthrobacter* sp.	*Micrococcus* sp.
Aedes taeniorhynchus	515	2	3						43				3	37		16	48						39							
	520	1			1				42							40														
	112		15		2					47																				
	226				100								23				1													
	263	30																												
	359	6			91				12				32																	
	322	33			22				20																					
	406				74																									6

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Azomonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Azotobacter* sp.	*Pseudomonas aureofaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles crucians	33					90																				10				
	31							3						97																
	185					54					13				33															
	318		94														6													
	20														100															
	704				44					14					42															
	376													99				1												
	22					100																								
	500		60	20														20												
	858	25	35	18		22																								
	192			100																										
	190	100																												
	210			100																										
	340								88																		12			

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Aeromonas* sp.	*Pseudomonas* sp.
Anopheles crucians	495		60	16								24				
	300		33		33							34				
	346		87								6					
	162		80								7					
(continued)	249			17	8				51							
	423		70	23							6					
	606		50			49										
	530		53			40						2				
	131			72												
	520									100						
	220									100						
	500		50								50					
	850			65												
	30		93													
	138		65	10												

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Azotobacter* sp.	*Pseudomonas cuneofaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles crucians	495														
	300														
	346										7				
	162	13													
(continued)	249	24													
	423									2					
	606		1												
	530														
	131		6									22			
	520														
	220														
	500														
	850											35			
	30										7				
	138		25												

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Sphaerella* sp.	*Corynebacterium* sp.	*Micrococcus pneumoniae*	*Saccharomyces* sp.	*Achromobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Neisseria rosea*	*Staphylococcus epidermidis*	*Aeromonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Flavobacterium* sp.	*Arthrobacter* sp.	*Pseudomonas aureofaciens*	*Acinetobacter* sp.	*Enterobacteriaceae*	*Klebsiella* sp.
Anopheles crucians	382														78			22											
	566	44	53															3											
	150															100													
	206																			61									
	500			62						38																			
	315			3						97																			
(continued)	22										73																		
	600			50							50																		
	155			3				87			10																		
	300	100													41		50	59											
	726																	50											
	600								100																				
	220																												
	380														100														
	365					32			16																				

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Moraxella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Aeromonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Acetobacter* sp.	*Pseudomonas aureofaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles crucians (continued)	610		49	2		49																								
	300		100																											
	--	100																												
									100																					

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Azomonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Azotobacter* sp.	*Pseudomonas aureofaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles quadrimaculatus (colony)	300	100																												
	350	36					14																							
	300	100																												
	309	97																			3									
	150	99					1																							
	269	93																												7
(continued)	300	100																												
	142	100																												
	150	100																												
	354	100																												
	150	100																												
	100	100																												
	200	100																												
	300	100																												

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Azomonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Azotobacter* sp.	*Pseudomonas coronafaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles quadrimaculatus (colony)	100	82					12																							
	120																				100									
	200	56																				43								
	25																100													
	130																				100									
(continued)	125	100																												
	300	100																												
	300	50					50																							
	250	100																												
	300	100																												
	300	100																												
	150	20												30																
	200	100																												
	300						100																							
	250	100																												
	312	96					4																							

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Eatmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomycetes* sp.	*Enterobacter aerogenes*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Azomonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Flavobacterium* sp.	*Aeromonas* sp.	*Arthrobacter aurocyaneus*	*Neisseria* sp.	*Flavobacterium luteum*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles																														
quadrimaculatus	325	100																												
(colony)	200	100																												
	100	100																												
	120	100																												
(continued)	90	100																												
	173	86																												

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Aeromonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Azotobacter* sp.	*Pseudomonas cancofaciens*	*Neisseria* sp.	*Flavobacterium lutescens*	*Arthrobacter* sp.	*Micrococcus* sp.
Anopheles crucians complex	162			93					2																					
	300											100																		
	300											100																		
	124		20																									80		
	330		10			90																								
	420		29					71																						
	270												37							60		3								
	142		35	65																										
	300							100																						
	535	56		11																								33		
	4				100																									
	230	13																		87										
	312	96			4																									
	411			27						73																				
	22					100																								
	331							91														9								
	10			100																										
	300								100																					

TABLE III-1 (continued)

SPECIES COMPOSITION (PERCENT OF TOTAL)

MOSQUITO SPECIES	TOTAL BACTERIAL COUNT	*Bacillus cereus*	*Alcaligenes faecalis*	*Salmonella* sp.	*Corynebacterium* sp.	*Klebsiella pneumoniae*	*Saccharomyces* sp.	*Enterobacter cloacae*	*Micrococcus luteus*	*Lactobacillus* sp.	*Escherichia coli*	*Proteus vulgaris*	*Micrococcus roseus*	*Staphylococcus saprophyticus*	*Aeromonas* sp.	*Pseudomonas* sp.	*Acinetobacter calcoaceticus*	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Streptococcus* sp.	*Citrobacter freundii*	*Planococcus* sp.	*Acinetobacter* sp.	*Pseudomonas* [illegible]	[illegible] sp.	*Flavobacterium* [illegible]	*Arthrobacter* sp.	[illegible] sp.
Anopheles quadrimaculatus (wild)	10	99																												
	280	18																	82											
	2407		5					37												58										
	910			1																										99
	3000										70																		30	
	32							100																						
	170							100																						
	300																							100						
	120																							100						
	300		100																											
	45																								100					
	900							100																						

midgut sterility encountered. Twenty-five specimens however, had an average midgut bacterial count of 666. The most frequently isolated bacterial species were *Enterobacter cloacae* (Jordan) followed by *B. cereus*.

Anopheles crucians complex

Forty-three percent of this mosquito group were bacteriologically sterile. The average midgut bacterial count of 25 specimens was 272 which was usually composed of *Salmonella* sp., *Alcaligenes faecalis* Castellani and Chalmers, and *E. cloacae*.

Anopheles crucians Wiedemann

The midgut sterility rate of *An. crucians* was 36%. Eighty specimens however, had an average midgut bacterial count of 377. The most frequently isolated bacterial types were *B. cereus*, *Salmonella* sp., *A. faecalis*, and *Klebsiella pneumoniae* (Schroeter).

Anopheles quadrimaculatus Say (colony)

Colonized *An. quadrimaculatus* had a midgut sterility rate of 40%. Sixty individuals had an average midgut bacterial count of 214, which was composed of *B. cereus* and the yeast, *Saccharomyces* sp.

The Normal Flora

Salmonella sp. appeared most frequently in *An. crucians* and *An. crucians* complex. They produced very small (1-2 mm) mucoid, discrete colonies on Eugon agar and blood. A negative Gram stain demonstrated motile, non-encapsulated rods. Most isolates were catalase positive

and all were able to utilize citrate. Decarboxylase reactions were variable, but most isolates carboxylated lysine and ornithine, and produced H_2S on TSI. Carbohydrate fermentations were quite variable. Antigenic typing of *Salmonella* sp. was not attempted.

The Actinomycetales were represented by a group of non-pathogenic *Corynebacterium* and the genus *Arthrobacter*. The former were frequently isolated from *A. infirmatus*, while the latter appeared only in a single isolate from a wild *An. quadrimaculatus*. The *Corynebacterium* sp. produced grey to white star-shaped colonies on Eugon agar and frequently produced luxuriant growth on blood agar without hemolyzing it. These isolates were characterized by their high degree of pleomorphism which usually took the form of a non-spore forming Gram-positive rod of irregular outline. Most isolates were catalase positive and produced strong oxidative reactions on OF media. These isolates were presumed to be non-pathogenic coryneforms because of their apparently non-pathogenic nature.

A. faecalis was isolated most frequently from *An. crucians*. They produced pale yellow wrinkled colonies on Eugon's agar. They usually occurred as motile coccoid Gram-negative rods. Most isolated produced no change on TSI slants and negative reactions on indole, H_2S and urea slants. Carbohydrate fermentations were also negative. Simmons-citrate reactions were variable, but all isolates produced oxidative type OF reactions.

E. cloacae was isolated from nearly 50% of wild *An. quadrimaculatus* examined. These Gram-negative motile rods usually produced smooth, round, rose-pigmented colonies on Eugon agar. They were distinctive

in producing an acid TSI, positive citrate and a positive VP test. Most isolates produced moderate to strong hydrolytic reactions on starch agar, lipid agar, and gelatin agar. An occasional strain hydrolyzed cellulose (5% alphacel[1]). Catalase reactions were positive and most carbohydrates were actively fermented. Nitrates were reduced by most isolates.

B. cereus was the dominant midgut bacterial species of 95% of the colonial *An. quadrimaculatus* that were contaminated. It appeared occasionally in all other mosquito species. *B. cereus* produced rough, white lobate or round colonies on Eugon' agar. Their long, thick rods usually occurred in long chains and produced cylindrical spores. All isolates were catalase positive and produced strong hydrolytic reactions on starch agar and gelatin agar. Decarboxylase activity was usually not present. Carbohydrate fermentations were variable, but arabinose, xylose, and mannitol typically were not fermented. *B. cereus* was differentiated from *Bacillus subtilis* (Ehrenberg) which was occasionally isolated from colonized *An. quadrimaculatus* by their inability to form chains and their characteristic litmus milk reaction.

K. pneumoniae was isolated most frequently from *An. crucians*. They produced 1-2 mm brown colonies on Eugon agar and appeared as non-motile capsulated rods which occur singly or in short chains. Occasionally, the fimbriae were recognized. This group typically produced negative reactions in ornithine, and positive reactions in gelatin. They produced acid and gas from glucose, utilized citrate, and produced a positive VP reaction. These isolates often produced luxuriant growth with a green non-hemolytic sheen on blood agar.

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[1] Nutritional Biochemicals Co., Cleveland, Ohio

Micrococcus roseus Flugge and *M. luteus* were isolated most frequently from *A. infirmatus* and *An. crucians*, respectively. They are Gram-positive cocci that produced large, opaque growth on Eugon's agar under aerobic conditions. Cell division occurred in 2 or more planes forming irregular clusters. Both groups were strongly catalase positive and produced acid without gas from glucose. *M. roseus* hydrolyzed starch and gelatin, and produced bright red colonies on lipid agar. Smooth, convex rose-colored colonies were produced on Eugon agar. Metabolism was of the respiratory type and decarboxylose activity was negative. *M. luteus* produced yellow, glossy colonies on Eugon agar and was distinguished by its ability to hydrolize fats. An unidentified non-pigmented *Micrococcus* sp. was occasionally isolated from wild *An. quadrimaculatus* which failed to hydrolize starch and gelatin, and produced variably positive decarboxylase reactions.

Pseudomonas aeruginosa (Schroeter) and *Pseudomonas aureofaciens* Kluyver were isolated in low frequency from *A. infirmatus* and *An. crucians*, respectively. An unidentified *Psuedomonas* sp. was isolated 3 times from *A. crucians*. This family of bacteria are short, motile, Gram-negative rods that are highly pigmented. *P. aureofaciens* produced bright orange discrete colonies while *P. aeruginosa* developed red pigment. Neither species gave a positive MR, VP, or H_2S reaction. Negative decarboxylase reactions occurred in lysine, ornithine, and arginine medias. Both species gave alkaline milk reactions and produced strong catalase reactions. Nitrate reduction was variable.

Serratia marcescens Bizio was isolated from wild *An. crucians* complex. These bacteria were motile, Gram-negative rods that produced smooth 2-3

mm colonies on Eugon agar. They gave strong catalase reactions and reflected a greenish-iridescence in oblique light. Most isolated produced brigh red colonies on lipid agar and hydrolized starch agar. They produced positive VP reactions and were able to utilize citrate. Metabolism was of the fermentative type.

Escherichia coli (Migula) was isolated frequently from *An. crucians* and *An. crucians* complex. These Gram-negative rods produced acid and gas but no H_2S on TSI. No capsules or fimbriae were noted, but isolates were strongly catalase positive. Ornithine, lysine, and arginine are decarboxylated. Citrate was not utilized but acid and gas were produced from xylose, dextrose, maltose, lactose, and mannitol. Antigenic typing was not performed.

Frequent isolates of *Staphylococcus saprophyticus* (Fairbrother) were made from *An. crucians*. These Gram-positive cocci occurred in clusters and produced smooth, white convex colonies on Eugon agar. They were facultatively anaerobic with strong catalase reactions and variable decarboxylase activity. Acid was produced from glucose, lactose, and sucrose. Nitrates were reduced and gelatin, lipids, and urea were hydrolyzed. Citrate could be utilized as a sole source of carbon.

Streptococcus faecalis Andrews and Harper and an unidentified *Streptococcus* sp. were isolated from *An. crucians*. These Gram-positive cocci typically occurred in pairs or in short chains. Metabolism was of the fermentative type and catalase tests were negative. Colonies appeared as discrete, white and mucoid on Eugon agar and luxuriant on blood agar. *S. faecalis* did not hemolyze blood, but the unidentified *Streptococcus* produced strong beta hemolysis. An acid curd with reduction appeared in

litmus milk and nitrates were not reduced. Both citrate and glucose were actively fermented. Both cultures grew in the presence of .04% tellurite. Antigen typing was not accomplished.

An unidentified isolate showing the characteristics of *Lactobacillus* sp. was consistently isolated from *An. crucians* complex. These Gram-positive, non spore-forming rods or coccobacilli form minute colorless colonies on Eugon agar. They were consistently catalase negative and failed to reduce nitrates. Indole, H_2S, acetyl methylcarbinal, and urea were not produced. They produced arginine dehydrolase but did not split lysine or ornithine. Proteolysis was often produced in litmus milk. Carbohydrate fermentation was variable.

Acinetobacter calcoaceticus (Beijerinck) was isolated consistently, but in low numbers from all 5 mosquito groups. These short, thick Gram-negative rods produced discrete smooth mucoid colonies on Eugon agar and brown hemolytic colonies on blood agar. These isolates produced a proteolytic reaction in litmus milk, were catalase positive, and failed to produce indole, H_2S, reduce nitrate or liquify gelatin. All isolates were oxidase negative and demonstrated weak OF reactions.

Three isolates from *An. crucians* were tentatively identified as a species of soil *Neisseria*. These Gram-negative cocci were aerobic and formed large, yellow colonies on Eugon agar and were hemolytic on blood. They were catalase and oxidase positive, and they produced indole, and acety methylcarbinal. They utilized citrate as a sole carbon source but failed to decarboxylate lysine, ornithine or arginine. Glucose and maltose were weakly attacked.

TABLE III-2

Correlation Coefficients of the Frequency of Occurrence of Midgut Bacterial Species

Species	R^1	P^2 >
Positive Correlation		
Neisseria sp.; *Salmonella* sp.	0.334	.0002
Azomonas sp. ; *S. faecalis*	0.272	.001
P. aureofaciens; A. faecalis	0.468	.0001
S. faecalis; A. faecalis	0.215	.001
A. faecalis; M. lutea	0.214	.012
S. lutea; Salmonella sp.	0.379	.0001
A. faecalis; P. aeruginosa	0.177	.037
Negative Correlation		
B. cereus; Salmonella sp.	-0.199	.001
B. cereus; A. faecalis	-0.264	.002
B. cereus; E. cloacae	-0.173	.004
B. cereus; K. pneumoniae	-0.180	.003

[1]Correlation coefficient
[2]Probability

The remaining bacterial species consisted of low frequency isolations or solitary isolations. These bacteria were *Proteus vulgaris* Hauser, *Flavobacterium lutescens* (Migula), *Citrobacter freundii* (Braak), and unidentified species of *Planococcus*, *Azobacterium*, and *Azomonas*. The yeast *Saccharomyces* sp. was identified by morphological characteristics and its ability to grow on Sabouraud maltose agar.

Correlation in Bacterial Occurrence

Table III-2 represents correlation coefficients and their probability statements calculated for each combination of bacterial species in the midgut flora. *Salmonella* sp. is positively correlated ($P>.001$) with both *Micrococcus luteus* and the unidentified *Neisseria* sp. *Streptococcus faecalis* is positively correlated ($P>.001$) with *Azobacter* sp. and *A. faecalis* although these combinations occurred in only 3 insects. *A. faecalis* is positively correlated with *M. luteus* ($P>.001$) and *P. aeruginosa* ($P>.005$).

The negatively correlated bacterial pairs all involved *B. cereus*. *B. cereus* is negatively correlated with *Salmonella* sp. ($P>.001$), *A. faecalis* ($P>.001$), *E. cloacae* ($P>.005$) and *P. pneumoniae* ($P>.005$).

Discussion

The large proportion of bacteriologically sterile individuals is not surprising. Chao and Wistreich (1959) reported a sterility rate of 8% and 23%, respectively, for adult female and male *Culex tarsalis* Coquillette. Wistreich and Chao (1963) reported a midgut sterility rate of 24% and 40%, respectively, for female and male adult *Aedes aegypti* L. Micks et al. (1961) reported midgut sterility rates of 50% for *An. quadrimaculatus* females, 91% for *A. aegypti*, and 78% and 89%, respectively,

for *Culex fatigans* Wiedemann and *Culex molestus* Forskal. Micks and Ferguson (1963) in a later investigation, reported the midguts of all *C. molestus* females examined were uniformly sterile.

The presence of a bactericidal and/or bacteriostatic substance in the midgut of certain mosquito species has some support. Duncan (1926) demonstrated the presence of a bactericidal substance present in the gut contents and feces of *Aedes cinereus* Meigen and *Anopheles bifurcatus* (L) which was effective against 3 species of *Bacillus*. More recently, the activity of antibacterial factors present in other blood-sucking arthropods has been largely associated with and limited to Gram-positive bacteria (Duncan, 1926; Anigstein et al., 1950).

The fact that microorganisms could not be isolated from a number of the specimens in this and other studies should not be taken to mean that microorganisms were not there. The standard cultural procedures employed in these studies are not adequate to demonstrate intracellular or exquisitely fastidious organisms.

The species composition of the microflora isolated from the 5 groups of mosquitoes in this study compares favorably with other workers. The isolates from adult *C. tarsalis* (Chao and Wistreich, 1959) showed the presence of *Achromobacter gutatus* (Zimmerman). *Achromobacter* sp., *E. cloacae*, *Citrobacter intermedia* (Braak), *Flavobacterium* sp., *Micrococcus* sp., *Micrococcus virians* Migula, *Proteus* sp., and *Pseudomonas* sp. Cultures of unidentified Gram-negative rods as well as *Saccharomyces* sp. and *Geotrichum* sp. were also reported. The same investigators, Wistreich and Chao (1960), isolated bacteria from midgut sections of 14 fourth-instar larvae of *C. tarsalis* that had been raised in fresh tap water and fed

brewer's yeast. Results of these isolations showed the presence of *Achromobacter* sp., *Lactobacillus* sp., *Micrococcus candidus* Cohn, *Micrococcus* sp., *Proteus rettgeri* Hadley et al., and *Pseudomonas* sp. Again, cultures of unidentified Gram-negative rods and *Saccharomyces* sp. and *Geotrichum* sp. were observed. Isolations from the larval rearing water showed the presence of *E. cloacae*, *Klebsiella* sp., *Saccharomyces* sp. and *Geotrichum* sp. Fourth instar larvae were found to contain examples of all the genera of bacteria isolated from adult *C. tarsalis* with the exception of *Enterobacter*, *Citrobacter*, and *Flavobacterium*, whereas isolations from adults did not show the presence of *Lactobacillus* or the coral pigmented form of *Micrococcus*.

Bacteria isolated from the midguts of adult *Culex quinquefasciatus* Say fed apple slices for 2 days after emergence showed the presence of *Achromobacter* sp., *Flavobacterium* sp., *Klebsiella* sp., *Micrococcus caseolyticus* Evans, and *Pseudomonas* sp. (Chao and Wistreich, 1960). Midgut sections from 11 fourth instar larvae of the same species produced only *Bacillis* sp. and the yeast *Saccharomyces* sp.

Bacteriologic evaluation of the midguts of larval and adult *Aedes sierrensis* Ludlow and *Anopheles albimanus* Wiedemann fed on a larval diet of yeast and adult diet of apple slices was reported by Wistreich and Chao (1961). Larval isolation from *A. albimanus* showed the presence of *Alcaligenes* sp., *Bacillus* sp. and unidentified Gram-negative rods. Adult *A. albimanus* showed *Achromobacter* sp., *Alcaligenes* sp., and *Corynebacterium* sp. *Saccharomyces* was isolated from both larvae and adults. *A. sierrensis* larvae contained *Bacillus circulans* Jordan, and *Paracolobactrum intermedium* Borman. *Achromobacter* sp. and *C. intermedia* were found in adults. *Sac-*

charomyces sp. were isolated only from the larvae. Wistreich and Chao (1961) also reported that microorganisms isolated from the adult and larval environments were distinctly different from isolates of the midguts.

Wistreich and Chao (1963) reported bacteriologic evaluations on the midguts of larval and adult *A. aegypti* which had been fed yeast and apple slices, respectively. Isolations from larvae showed the presence of the following bacteria: *Alcaligenes* sp., *Corynebacterium* sp., *C. freundii*, *Kurthia bessonii* Hauduroy et al., *M. luteus*, as well as fungi belonging to the generas *Geotrichum*, *Penicillium*, and *Saccharomyces*. Isolations from adult mosquitoes showed the presence of *Achromobacter* sp., *Alcaligenes* sp., and *Saccharomyces* sp.

Ferguson and Micks (1961) reported additional experiments using adult female *C. fatigans* from a colony that had been denied access to food. By means of a sterile micro-dissection technique, they demonstrated the presence of *Lactobacillus* sp., *Alcaligenes* sp., *Pseudomonas* sp., and 2 unidentified cultures of Gram-negative rods. Bacterial isolates from the midgut of similarly treated adult female *An. quadrimaculatus* proved to be 2 strains of *Streptococcus* and a species of *Aerobacter* (Micks and Ferguson, 1963). *A. aegypti* produced only a species of *Corynebacterium* and no microorganisms could be cultured from the midguts of adult female *C. molestus*.

Generally, these bacteria represent common soil, water, or enteric organisms widely distributed in nature. Four of the 6 collection sites from which *An. crucians*, *An. crucians* complex and wild *An. quadrimaculatus*

were collected were locations around a large lake on the University of Florida campus. This lake receives a number of sewerage effluent. The presence of such enteric organisms as *P. vulgaris*, *E. coli*, and *A. faecalis* in the midguts of detritis feeders is not surprising.

Colonial mosquitoes tend to exhibit a microflora made up of 1 or 2 bacterial types which are consistent for the colony. Wild mosquitoes usually have a variety of microorganisms which are generally comparable within the species.

A notable exception proved to be the isolation of *Neisseria* sp. from 3 specimens of *An. crucians*. They resembled the Micrococcaceae. somewhat, but bore an even closer resemblance to 2 types of *Neisseria* described in grasshoppers by Bucher and Stephens (1959). One isolate was tentatively identified as *N. catarrhalis* (Frosch and Kolle). The taxonomic position of these 2 types is uncertain and it is questionable if they belong to this family or if they should be considered a member of the Micrococcaceae that had lost their ability to retain the Gram stain or to ferment carbohydrates.

The significance of the positively correlated bacterial combinations is difficult to interpret. *S. faecalis* and *A. faecalis* might be expected to occur together because of their enteric origin. The other combinations may be the result of mutually satisfying metabolic relationships.

Significance of the negatively correlated combinations favoring the presence of *B. cereus* is more obvious. The family Bacillaceae produce a variety of extracellular products. Those products secreted by *B. cereus*

include hemolysin, soluble toxin lethal for mice, enzymes lytic for bacterial cells, proteolytic enzymes and phospholipase C. (Buchanan and Gibbons, 1974). *B. cereus* has been incriminated in food poisoning where extensive multiplication has occurred in foods. By way of its secreted products, this bacterial species apparently competitively inhibits most other intestinal microorganisms, with the yeast *Saccharomyces* being a notable exception.

RESULTS

IV. EFFECTS OF BACTERIOLOGICAL FLORA ON THE EARLY DEVELOPMENT OF DIROFILARIA IMMITIS LEIDY IN ANOPHELES QUADRIMACULATUS SAY

Introduction

The relationship of the normal intestinal microflora to the vectoring capacity of mosquitoes has not been adequately investigated. Insects are not unique among animals in their relationships with microorganisms. Parasites and potential parasites, passing through the arthropod hosts alimentary tract are exposed to both the microorgansism that reside there, and to the intestinal climate which may, in part, be mediated by them. Indigenous bacterial flora present at the host-parasite interface must be regarded with as much consequence as the host itself.

Dirofilaria immitis Leidy, the etiological agent of canine heartworm disease, requires passage through the mosquito to the L_3 or infective stage. Although susceptible or resistant mosquitoes species are genetically determined (reviewed by MacDonald, 1973), the physiologic mechanism whereby the insect accepts or rejects the infection has not been elucidated. The presence or absence of host microflora, or the species composition of microflora, may be one of the factors mediating the establishment of *D. immitis* in susceptible mosquitoes.

Gnotobiotic studies have shown that the success of many parasitic diseases depends not only on the etiologic agent, but also upon the host's

intestinal microflora. Phillips and Gorstein (1966) reported that the association of the amoeba *Entamoeba histolytica* Kruse) with one of 3 species of bacteria was necessary to produce typical hepatic abcesses in hamsters. Phillips (1964) considered the bacteria acted by providing a physically and chemically suitable environment favorable to survival of the amoeba, but Wittner and Rosenbaum (1970) proposed the existence of some critical factor passing from bacteria to pathogen. Micks and Ferguson (1961) concluded that microorganisms play a significant role in the susceptibility of mosquitoes to malarial parasites. They suggest either a competition between the microorganism and malarial parasite for essential nutrients, or that certain strains of microorganisms supply factors needed by the host for metabolic processes and defense mechanisms. Elimination of the microorganisms in the latter case would presumable increase host susceptibility to malaria.

This paper reports the possible effects of midgut bacterial flora isolated from 5 groups of wild and colony mosquitoes on the ability of gnotobiotic mosquitoes to support development of first stage *D. immitis* larvae.

Materials and Methods

Eggs of *Anopheles quadrimaculatus* Say were obtained from the colonies of the USDA Agricultural Research Service, Insects Affecting Man Laboratories in Gainesville, Florida. Egg sterilization procedures, larval diet, and germ-free rearing techniques have been previously described (Results II). Eggs were surface sterilized in 10% Zephiran; allowed to hatch, and the sterile larvae injected into 16 ounce prescription bottle rearing

flasks containing a high protein larval diet. After 8 days incubation, sterile pupae were withdrawn and injected into a sealed lucite capsule designed for the maintenance of gnotobiotic, blood-feeding arthropods (Results II). Emerging adults were maintained on sugar solution for several days before receiving an infective blood meal through a heated artificial membrane built into the capsule.

Concurrently, a quantitative and qualitative bacteriologic evaluation was being compiled on the midguts of several hundred adult female specimens of colonized *An. quadrimaculatus*, wild *An. quadrimacualtus*, *Anopheles crucians* Wiedeman, *Anopheles crucians* complex, and *Aedes infirmatus* Dyar and Knab (Results III). Midguts were dissected from surface sterilized female specimens, individually homogenized, and plated onto Eugon [1] agar and blood fortified Eugon's[1] agar. A total count of all colony-forming units produced in 24 hours of incubation, and a differential count of each colony type was made from each midgut preparation. Pure cultures of each colony type was identified according to standard technique.

The total midgut bacterial count, and the species composition of midgut flora was distinctive for each group of mosquitoes. The most frequently isolated bacterial species for each group of mosquitoes were: *A. infirmatus*, non-pathogenic *Corynebacterium* sp., wild *An. quadrimaculatus*, *Enterobacter cloacae* (Jordan), and *Acinetobacter calcoaceticus* (Beijerinck), colonized *An. quadrimaculatus*, *Bacillis cereus* Frankland and Frankland, *An. crucians*, *Alcaligenes faecalis* Castellani and Chalmers, and *An. crucians* complex, *Salmonella* sp. Pure cultures of these bacteria were

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[1] Difco Laboratories, Detroit, Mich.

subsequently inoculated into larval culture flasks to produce gnotobiotic mosquito larvae monocontaminated with a major bacterial isolate of a normal wild mosquito. Rearing proceeded normally, and a sample of the adults emerging on the 10th day proved to be infected with a single bacterial type.

After a 3 day holding period, 6 groups of 10-15 monocontaminated females each, a group pf 15 gnotobiotic females, and 25 conventional specimens taken from the colony, were given a blood meal containing 66,000 *D. immitis* microfilariae per ml. The monocontaminated specimens, and the gnotobiotic specimens, were fed through a heated artificial membrane in their capsule. Conventional mosquitoes were fed on the shaved hindquarters of the same donor dog which provided the infected blood for the gnotobiotic mosquito groups. All mosquito groups were held 48 hours in their capsule before being dissected. The degree of filarial development was recorded by light and phase photomicroscopy, and typed according to a procedure developed by Sawyer and Weinstein (1963a). These classifications were : Type Ia - typically unchanged; 285-300μ long and 4.5-6.0μ wide. Type Ib - uniform increase in width but only slightly different from Ia. Type II - significantly widened with a decrease in length, typically wider posteriorly than anteriorly; 240-300μ in length, 6.0-9.0μ in width. Type III - "pre-sausage" with distinct increase in width (6.0-12.0μ) and decrease in length (190-230μ). Type IV - "Sausage-form", 150-200μ long, and 9.0-12.0μ in width. Larval measurements are expressed as the averaged length and width measurements of 10 specimens in one microscopic field.

Results

Figure 1 represents a Malpighian tubule "squash" from a conventional *An. quadrimaculatus* 48 hours after taking an infective blood meal. The first stage larvae pictured are essentially undeveloped Type I and Ib which measure an average of 292μ x 6.5μ. General development appears to be consistent with the description of *D. immitis* larvae after 2 days development in *Aedes aegypti* (L) reported by Taylor (1960). The bacterial flora of conventional, colony-reared *An. quadrimaculatus* used in this study consisted almost entirely of *B. cereus*.

Figure 2 illustrates another essentially unchanged Type I larva recovered from female *An. quadrimaculatus* monocontaminated with a species of *Salmonella* originally isolated from wild *An. crucians* complex. These larvae averaged 289μ x 6.5μ and were indistinguishable from larvae undergoing development in conventional mosquitoes.

The larva pictured in Figure 3 correspond to Sawyer and Weinstein's (1963a) early Type III development. These larvae measured an average of 256μ x 7.5μ and showed an advanced development at 48 hours quite comparable to Taylor's (1960) description of 2nd-3rd day larvae. They were recovered from a female *An. quadrimaculatus* monocontaminated with *A. calcoaceticus* originally isolated from wild *An. quadrimaculatus*.

Figure 4 illustrates Type II larvae whose average measurement was 240μ x 8.0μ. These larvae are typical of those recovered from mosquitoes monocontaminated with *B. cereus* originally isolated from colony-reared *An. quadrimaculatus*.

Slightly advanced development is noted in Figure 5. These larvae measured 230 x 8.3 and correspond to Sawyer and Weinstein's (1963a) early Type III development. They developed in a mosquito monocontaminated with *Lactobacillus* sp. originally isolated from wild *An. crucians*.

An example of the encapsulation reaction, a manifestation of host resistance is illustrated in Figure 6. This Type I-Ib larva is an example of several encapsulated larvae recovered from *An. quadrimaculatus* monocontaminated with a species of non-pathogenic *Corynebacterium* sp. originally isolated from *A. infirmatus*.

Figures 7 and 8 represent typical "sausage-form" Type IV larvae comparable to Taylor's (1960) description of 4-5 day old *D. immitis* larvae in *A. aegypti*. These larvae, which were recovered from gnotobiotic mosquitoes, measured 160 x 11.0 and manifest an advanced state of development. This degree of development at 48 hours was seen only in larvae recovered from gnotobiotic mosquitoes. The relative size of these larvae is evident in Figure 8 which shows 2 sausage-form larvae within a single tubule.

A proportion of bacteriologically sterile individuals were encountered in 4 of the 5 mosquito groups. Sixty-one percent of the wild *An. quadrimaculatus* were sterile while 40% of the colonized *An. quadrimaculatus* were sterile. *An. crucians* and *An. crucians* complex were 36% and 43%, sterile. All specimens of *A. infirmatus* examined were bacteriologically sterile.

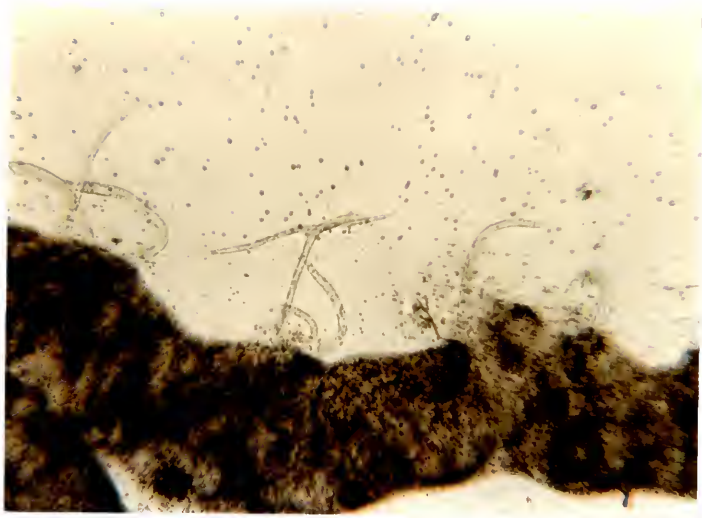

Figure IV-1. Type I-Ib Larva in Malpighian Tuoule "Squash" From a Conventional Mosquito 48 Hours After Taking an Infective Blood Meal (100x).

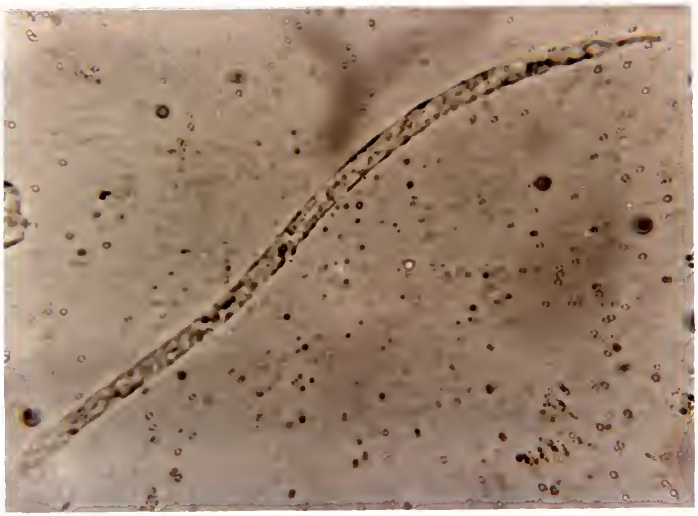

Figure IV-2. Typically Unchanged Type I Larva Recovered After 48 Hours in a Mosquito Monocontaminated with *Salmonella* sp. (400X).

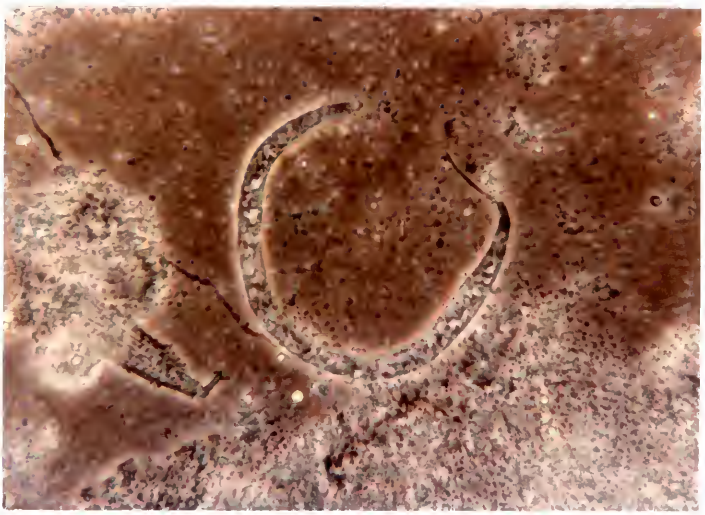

Figure IV-3. Early Type II Larva Recovered After 48 Hours in a Mosquito Monocontaminated with *Acinetobacter calcoaceticus* (400x).

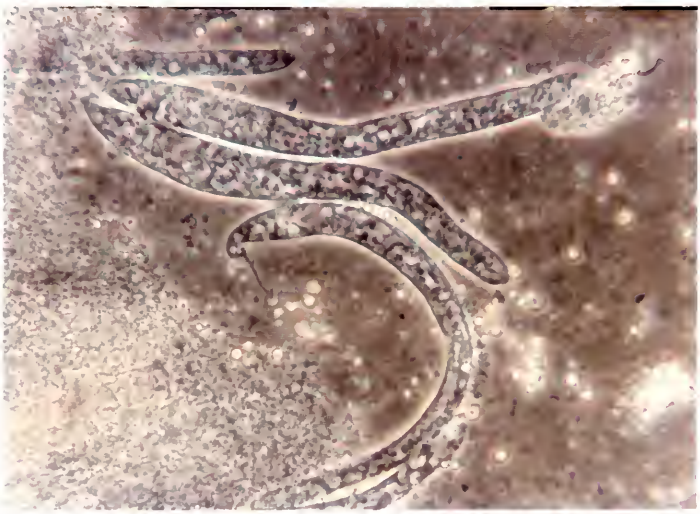

Figure IV-4. Type I Larvae Recovered After 48 Hours in a Mosquito Monocontaminated with *Bacillus sphaericus* (400x).

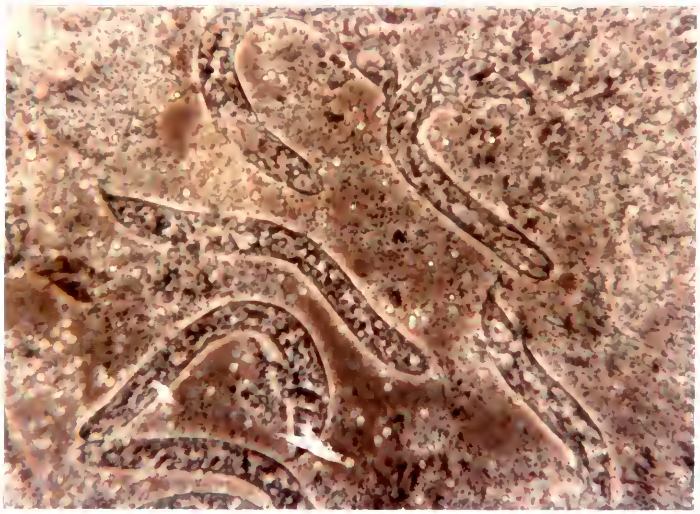

Figure IV-b. Early Type III Larvae Recovered After 48 Hours in a Mosquito Noncontaminated with *Lactobacillus* sp. (400x).

Figure IV-6. An Encapsulated Type I Larva Recovered After 48 Hours in a Mosquito Monocontaminated with Non-pathogenic *Corynebacterium* sp. (400x).

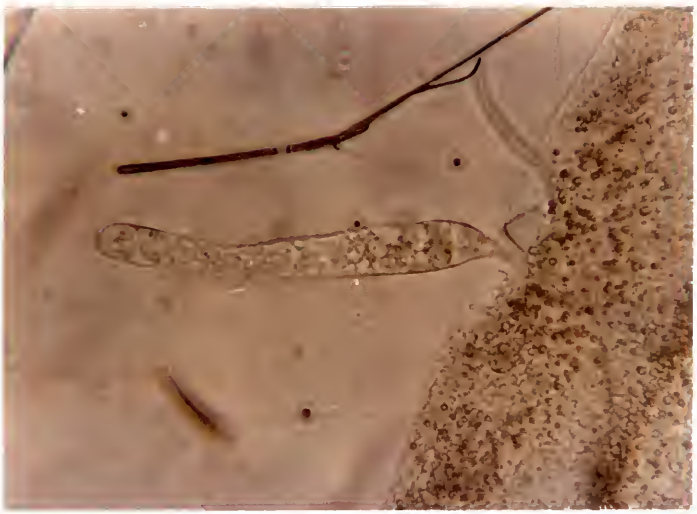

Figure IV-7. Typical "Sausage" Stage Type IV Larva Recovered After 48 Hours in a Gnotobiotic Mosquito (400x).

Figure IV-8. Two "Sausage" Stage Type IV Larvae Within a Tubule of a Gnotobiotic Mosquito (400x).

Discussion

It should be noted that larval development was not entirely synchronous in any mosquito. A range of developmental types was always present and the observer found it necessary to make an interpretation of which larvae were most typical of a midgut preparation. Ten typical larvae within a microscopic field were then measured and an average dimension interpreted with Sawyer and Weinstein's (1963a) criteria.

Allowing this degree of interpretation, it may be concluded that the early development of *D. immitis* larvae proceeds independently of bacteria or bacterial mediation in the midgut. "Sausage" stage larvae appeared within the first 48 hours in gnotobiotic mosquitoes, but required at least 72 hours in conventional mosquitoes. Nematode development appears to be marginally improved in the complete absence of bacteria and differentially retarded in the presence of pure cultures of certain bacterial species. Pure cultures of *Lactobacillus* and *B. cereus* were found associated with more advanced larva, while *Salmonella* sp., *A. calcoaceticus* and *Corynebacterium* sp. monocontaminants were found in the presence of undeveloped larvae.

If the presence of midgut bacterial contamination is inversely related to the development of *D. immitis* in the mosquito, a parallel might be drawn with *Leishmania* infected Phlebotomid flies. Alder and Theodor (1957) reported that the alimentary tract of sandflies must be bacteriologically sterile to successfully transmit leishmania. A chance bacterial contamination in a normally sterile intestine, interferes with the digestion of the blood meal and is fatal to the insect. Gnotobiotic mosquitoes, however, are fully capable of digesting their blood meal, as shown in this study.

A high percentage of sterile midguts must exist in nature. Wistreich and Chao (1963) reported 80% sterility in colonized *A. aegypti* which compares to 40% and 61%, respectively, for colonized and wild *An. quadrimaculatus* in the present study. If sterile midguts produce advanced *D. immitis* earlier, it is apparent that the "naturally" sterile specimens in the field will be the most effective vectors.

Bacterial mediation of vector efficiency may manifest itself in the physical or chemical control of the host midgut. For example, *Salmonella* sp., *A. calcoaceticus,* and *Corynebacterium* sp. are all associated with poorly developed larvae and all produce culture medias with a pH above 5.3. *B. cereus,* on the other hand, is associated with more advanced larvae, and produces a culture media below pH 5.3.

Various products of bacterial metabolism, changes in the redox potential or even toxic factors may be produced by indigenous bacteria to render the intestine inhospitable for filarial development. The passage of filarial larvae through the arthropod gut evidently requires a critical set of conditions since attempts to culture infective larvae *in vitro* have met with little success (Weinstein, 1970, 1972; Rothstein and Brown, 1960; Sawyer and Weinstein, 1963a, 1963b; Taylor, 1960; Wood and Suitor, 1966; and Cupp, 1972). Future investigations into this "critical set of conditions" within the arthropod intestine will be severely compromised without due consideration to the indigenous bacterial microflora which reside there.

DISCUSSION AND CONCLUSIONS

One of the commonly held generalizations is that blood feeding arthropods have sterile intestines, or, if they become contaminated while feeding, they are able to rid themselves of bacteria (Steinhaus, 1942; Weyer, 1960). Mosquitoes are bacteriologically sterile on hatching (Dougherty, 1959) but soon acquire a bacteriologic flora as indiscriminant detritis browsers and filter feeders. This fortuitously acquired flora may persist throughout life or be lost as a result of the structure of the alimentary tract or the incomplete nature of vertebrate blood and plant sugar diets. This is in contrast to such insects as grasshoppers which are sterile upon hatching, but soon acquire a bacterial flora which increases both in absolute numbers and in variety of species as the insect develops (Bucher, 1960).

The anatomy of the mosquito gut is an important factor in regulating the fate of microorganisms (Steinhaus, 1947, 1949). Straight tube forms of the alimentary tract typically contain only adventitious and saprophytic microorganisms. The complex type of gut with pouches, sacs, ceca, diverticula, or folds, set up gradients of food material, pH, and redox potential. Microorganisms respond to these gradients by increasing in variety and complexity. Brooks (1963) speculates that there may be locally differentiated floras correlated with the microenvironment of histochemically differentiated cells as found in blow fly larvae (Waterhouse, 1955).

The paucity of bacterial stimulation from a varied diet is another factor regulating the population of intestinal microorganisms. Vertebrate blood, besides being sterile, is deficient in vitamins which is the basis for the belief that only those insects which feed on blood throughout their entire life span possess symbiotes. Plant nectar is undoubtedly nearly sterile which accounts for the disproportionately high rate of midgut sterility in male mosquitoes which feed exclusively on plant sugar (Chao and Wistreich, 1959).

The diluting action of feeding further depletes the total midgut bacterial count. Mosquitoes commonly excrete small amounts of fluid immediately after feeding (Kartman, 1953a) which may account for the loss of additional microorganisms. Another loss of dejecta and presumably, microorganisms, occurs several days after blood feeding when digestion is complete.

Most bacteria seem to lack the ability to multiply in the gut of insects. *P. aeruginosa* does not grow in the gut of grasshoppers and, in fact, disappears rapidly from the gut (Bucher and Stephens, 1959). Other species of *Pseudomonas*, of *Proteus* and of pathogenic cloaca Type B occur rarely in the gut of grasshoppers and do not appear to be capable of multiplication there (Buchner and Stephens, 1959). Though Heimpel (1955) found that strains of *B. cereus* multiplied in the midgut of the larch sawfly, Bucher (1960) considers that this bacteria does not multiply in the midgut of grasshoppers or several other insects. *S. marcescens*, on the other hand, does persist in the gut of grasshoppers. Bucher and Stephens (1959) consider that some multiplication must occur to maintain its numbers against the diluting action of food.

Inhospitable physical conditions of the arthropod gut is yet another factor which regulates bacterial populations. The high pH of lepidopterous midguts obviously inhibits a large proportion of microorganisms. The low redox potential prevailing in the gut of many insects could be another important factor. Waterhouse (1952a,b) gave redox potentials between -170 and -300 MV for the larval midguts of clothes moths, dermestids and mallophaga species and showed that these very reducing conditions aided the digestion of keratin. Bucher (1963) concludes that aerobic pathogens would probably starve for oxygen in the gut of most insects and this may account for their inability to multiply.

The effectiveness of these factors in limiting the microorganisms of the arthropod intestine is reflected in the high proportion of sterile individuals in this and other studies. Presumably, as mosquitoes age, gut deflorication proceeds until a condition of bacteriologic sterility prevails in the intestine.

The great variety of microorganisms and their marginally predictable distribution in this study, supports the "fortuitious acquisition" theory of their origin. Eaves and Mundt (1960) concluded that *Streptococcus* sp. were randomly distributed as a result of circumstantial contact among 26 species of adult insects. Lysenko (1959) reported a similar conclusion regarding the common occurrence of saprophytic *Corynebacterium* sp. and *Brevibacterium* sp.

The external environment however, determines which organisms are available for contamination. Wedberg et al. (1949) reported that 10 species of bacteria representing 5 families, were recovered from the feces of wild *Blaberus* fed Pablum^{R} for a week after capture. Briscoe et al.

(1961) reported that 7 species of bacteria were isolated from aseptically removed sections of gut from Pablum®-fed, insectary-reared *Blaberus*. These 7 species were all members of one family, the Enterobacteriaceae, of which only 2 were identical to those isolated by Wedberg et al. (1949).

The fecal flora of *Blaberus* has been reported to change over a period of time when these insects are maintained in an immobile position and force-fed a clean diet of sucrose, milk, and yeast extract (Wedberg et al., 1949). The number of bacterial species per insect decreased as a result of the limited diet, which minimized the chance ingestion of microorganisms. This situation is in contrast to the case of grasshoppers which became more prolifically contaminated with time while feeding in the field.

The nature of the food and the feeding habits of mosquito larvae are more than adequate to thoroughly contaminate them with microorganisms. On the basis of mouth part structure (Pucat, 1965), the method of feeding, and the larval habitat, Culicine larvae have been classified as filter-feeders, browsers, and predators (Surtees, 1959). Renn (1941) referred to the characteristic *Anopheles* method of feeding in which floating particles are drawn straight toward the mouth as "interfacial" feeding. An alternate method of feeding occasionally employed by these larvae is "eddy" feeding in which particles move in converging curved lines. A number of independent studies (Barber, 1927; Coggeshall, 1926; Senior-White, 1928) established the fact that *Anopheles* larvae feed indiscriminantly upon any food material available to them, collectively described as detritis. The recent discovery in larval rearing

water of an autophagostimulant which intensifies filtering and the rate of particle ingestion (Dadd and Kleinjan, 1974) would naturally increase the rate of microbial contamination.

There is ample evidence that the intestinal flora of mosquitoes is merely chance bacterial contamination, and does not represent a "highly integrated ecosystem" analogous to the vertebrate gut. The indifferent acquisition of microbial flora however, does not diminish their significance to the insect. These microbial flora may have a highly determinate effect on the capacity of an arthropod to vector certain parasitic diseases.

The conventional *An. quadrimaculatus* selected for study from the colonies contained a highly significant bacterial species in their midgut. *B. cereus* has been named a "potential pathogen" of insects by Bucher (1960) because of its highly proteolytic nature. The extracellular products secreted by *B. cereus* include hemolysin, soluble toxin lethal for mice, enzymes lytic for bacterial cells, proteolytic enzymes and phospholipase C (Buchanan and Gibbons, 1974). The normal inhabitant of the alimentary tract apparently competitively inhibits most other incoming microorganisms with the single exception of *Saccharomyces* yeast. A continuous bacterial challenge probably occurs with *B. cereus* effectively "sterilizing" the intestine, producing natural monocontaminants. It should be emphasized that this is the normal situation in colonial mosquitoes.

When microfilariae of *D. immitis* are introduced into this melange of bacterial flux, they are exposed to the same lytic secretions and toxins and it is not surprising when nematode larvae do not advance beyond stage I in the first 48 hours. The same reasons may explain why a filarial infection does not establish, and when the apparently "resistant" mos-

quito is encountered. Kartman's (1952) concept of host efficiency was advanced to express a ratio between the theoretical number of microfilaria ingested by a group of mosquitoes and the total number of developing larvae remaining in them after a specified time. This is a measure of individual susceptibility. Kartman (1952) found that all *D. immitis* microfilariae in the midgut between 24-48 hours are killed and digested in *C. quinquefasciatus* and *C. pipiens*, but some larvae survive 72 hours in *A. aegypti* and *A. albopictus* and 48 hours in *An. quadrimaculatus*. Although not correlated with bacterial contamination, Kartman's (1952) mosquitoes were colony-reared specimens fed a dog biscuit diet that may well have contained *B. cereus* contaminants.

When gnotobiotic mosquitoes were monocontaminated with *B. cereus*, and subsequently infected with *D. immitis*, slightly different results were noted. *D. immitis* larvae achieve a more advanced development to stage II in the first 48 hours. The gnotobiotic mosquito differs from the conventional mosquito by not representing a continuous bacterial challenge situation. There is no requirement for competitive inhibition in the gnotobiotic intestine, and consequently, less of a threatening environment for the establishment of a filarial infection. Although the same species of bacteria predominates in both situations, the conventional midgut and the gnotobiotic midgut must be very different in terms of the challenge they represent to invading pathogens. It is not surprising therefore, that *D. immitis* maintains stage I development in the conventional midgut, but achieves stage II development in the *B. cereus* monocontaminated midgut.

Monocontaminations of gnotobiotic mosquitoes with other bacterial species yielded more difficult results to interpret. The failure of

D. immitis larvae to achieve its potential stage III development in the first 48 hours may be due to bacterial reactions other than lytic products. *Salmonella* sp., *A. calcoaceticus*, and *Corynebacterium* all produce culture media with a pH above 5.3. Klein and Bradley (1973) reported a failure of *D. immitis* larvae to develop beyond stage I in *in vitro* cultures when the pH of the media was elevated above pH 6. *D. immitis* larvae may be responding to a pH, redox, or chemical gradient by exhibiting variable early development when exposed to these differing monocontaminants.

The advanced stage III larvae which develop in the bacteria-free gut may be taking advantage of an upopposed establishment in an entirely compatible environment. *An. quadrimaculatus* are genetically excellent hosts of *D. immitis* and rarely mount cellular defense reactions such as encapsulation. Under conditions of a hospitable midgut, with no bacterial competition or inhibition, *D. immitis* larvae achieved an advanced state of development in the first 48 hours. Such early development has not been reported in any other *in vivo* or *in vitro* investigations.

The relationship of a sterile intestine to the vectoring capacity of an arthropod is significant. Aging mosquitoes presumably follow a process of bacterial deflorication which approachs bacterial sterility. If nematode development is accelerated under bacteria-free conditions, it appears that the naturally sterile mosquito is the most efficient vector of *D. immitis*.

A critical examination of the individual mosquito has revealed a biological relationship with internal microflora that serves to distinguish the species and the individual. This biological relationship with micro-

flora is subject to change over time, and it appears to represent one of the factors mediating the individual susceptibility of an arthropod vector to filarial infection. While it seems unlikely at present that such a biological relationship with microorganisms may prove of economic value in the control of either nematode or mosquito, it does represent a fresh approach to parasitic disease transmission studies.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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